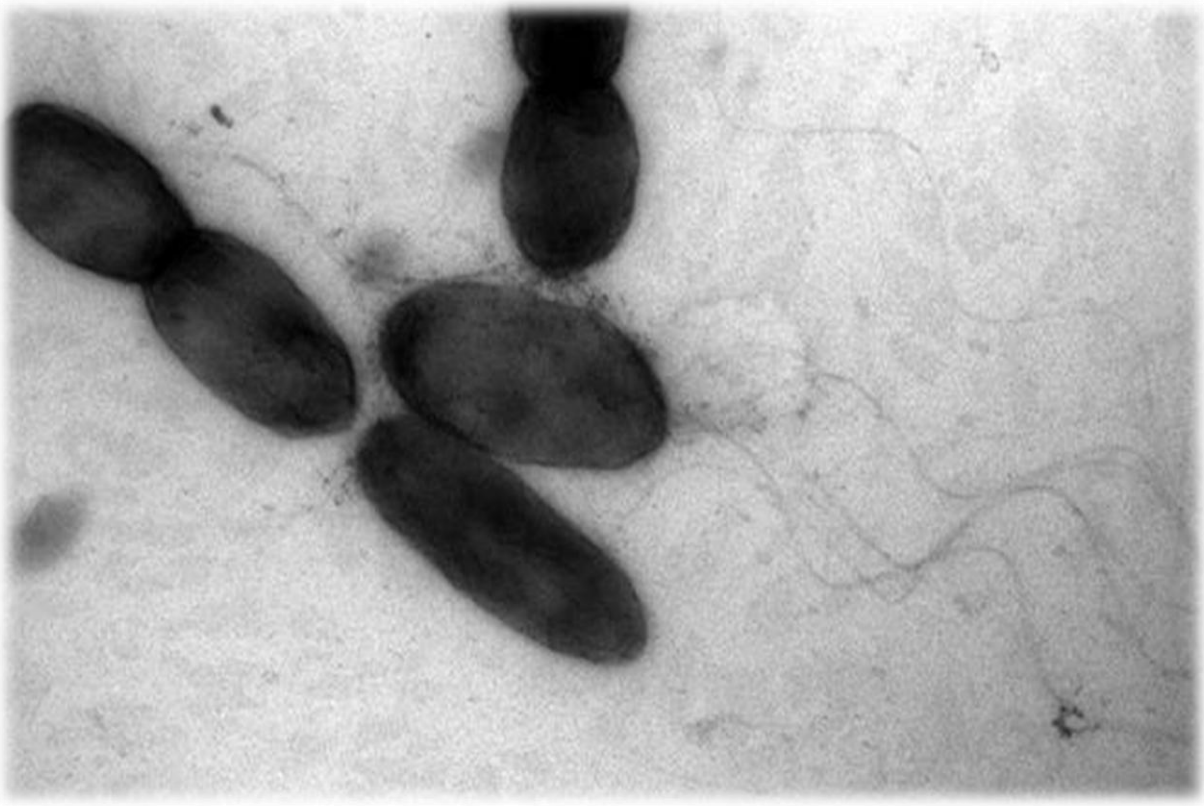
UNIVERSIDAD PABLO DE OLAVIDE SEVILLA

TESIS DOCTORAL

Characterisation of the response of *Sphingopyxis granuli* strain TFA to anaerobiosis



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Characterisation of the response of *Sphingopyxis granuli* strain TFA to anaerobiosis

Memoria presentada por
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First of all, I want to apologise, as most of my aknowledments are going to be in Spanish because, as it is my mother language, I think this way I can express better what I have felt and I still feel about this 5-years adventure.

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ABBREVIATIONS

AMP	Adenosine monophosphate
AOX	Alternative oxidase
ApR	Ampicillin resistance
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
cAMP	Cyclic AMP
cDNA	Complementary DNA
Cm	Centimetre
CmR	Chloramphenicol resistance
CoA	Coenzyme A
COG	Cluster of Orthologous Groups
CRP	Cyclic AMP receptor protein
CSPD	Chemiluminescent substrate for alkaline phosphatase
CTD	C-terminal catalytic domain
Cys	Cysteine
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dRNA-seq	Differential RNA Sequencing
DSB	Double strand DNA break
dsDNA	Duoble strand DNA
DSE	Double strand DNA end

EDTA	Ethylenediamine tetraacetic acid
EM	Electron microscopy
FAD	Oxidised flavin adenine dinucleotide
FADH2	Reduced flavin adenine dinucleotide
FIMO	Find Individual Motif Occurences
Fnr	Fumarate and nitrate reductase
FPKM	Fragments Per Kilobase Million
GmR	Gentamicin resistance
GSH	Glutathione
GSSH	Glutathione persulfide
GTE	Solution with glucose, tris-HCl and EDTA
H	Hour
HCO	Heme-copper oxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
Km	Michaelis constant
KmR	Kanamycin resistance
kV	Kilovolt
LB	Luria-Bertani medium
M	Molar
MEME	Multiple Em for Motif Elicitation
Min	Minute
MKH2	Menahydroquinone
mM	Millimolar
MM	Mineral medium
Mm	Milimetros
MML	Rich medium for TFA
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NER	Nucleotide excision repair
Ng	Nanogram
Nm	Nanometre
NTD	N-terminal DNA binding domain
ºC	Degrees Celsius
OD600	Optical density measured at 600 nm
PAGE	Poly-Acrylamide Gel Elctrophoresis
PB	B operon promoter
PBS	Phosphate-buffered saline
PBS-T	PBS with Tween-20
PC	C operon promoter
PCR	Polymerase chain reaction
PDO	Persulfide dioxigenase
PEG	Polyethylene Glycol
PH	Internal promoter in B operon, upstream of *thnH*

PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PM	M operon promoter
PMF	Proton motive force
PR	Internal promoter in C operon, upstream of *thnR*
Ptac	tac promoter
QH	Quinone
QH2	Quinol
RIC	Repair of iron centres
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolutions per minute
rRNA	Ribosomic RNA
RSSH/RSS-	Low-molecular weight persulfides
RT-qPCR	Real time quantitative PCR
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SmR	Streptomycin resistance
SmS	Streptomycin sensibility
SOB	Super Optimal Broth
SOC	Super Optimal broth with Catabolic repressor
SOD	Superoxide dismutase
SQR	Quinone oxidoreductase
SSC	Saline-sodium citrate
ssDNA	Single strand DNA
TAE	Tris Acetate-EDTA buffer
TB	Terrific Broth
TE	tris-EDTA
TER	Buffer with Tris-HCl, EDTA and ribonuclease
THN	Tetralin
TLS	Translesion synthesis
TSS	Transcription start site
TSS	Transformation and storage solution
UTR	Untranslated region
UV	Ultraviolet
V	Volt
v/v	Volume/volume rate
Vmax	Maximum velocity
w/v	Weight/volume rate
WT	Wild type
β-HB	β-hydroxybutirate
µL	Microlitre

ABSTRACT

Abstract

Sphingopyxis granuli strain TFA is an α-Proteobacteria isolated from the Rhine river that is able to degrade the organic solvent tetralin. Until now, the *Sphingopyxis* genus had always been described as strict aerobic, but we have demonstrated in this work that TFA is able to respire nitrate to nitrite in anaerobic conditions, thus becoming the first facultative anaerobic *Sphingopyxis* strain reported.

Two putative Fnr anaerobic regulatory proteins have been found in TFA, FnrN and FixK, which have shown to be necessary for anaerobic growth, being FnrN more relevant than FixK. Transcriptomic analyses of this bacterium by dRNA-seq and RT-qPCR have shown differentially regulated genes in anaerobiosis as compared to aerobiosis, which belong to different functional categories. Similar analyses using a double mutant Δ*fnrN*Δ*fixK* led to identification of genes directly regulated by the Fnr regulators. A regulon of 14 operons has been defined and consensus recognition site for these regulatory proteins, the Fnr box, has also been identified. The central metabolism of this bacterium is barely affected in anaerobic conditions, with scarce exceptions. As expected, different alternative terminal oxidases have been induced in anaerobic conditions: the nitrate reductase (*nar*), the cytochrome *bd* oxidase (*cyd*), the cytochrome *o* quinol oxidase (*cyo*), the cbb_3 cytochrome *c* oxidase (*cco*) and an NO-insensitive alternative oxidase (*aox*). However, only *nar* and *cco* showed Fnr sites and seemed to be regulated by Fnr. Interestingly, many genes involved in stress response and SOS repair systems have been induced in anaerobic conditions, suggesting that anaerobiosis is a hostile and mutagenic environment for TFA, probably due to nitrite accumulation as the respiration product and its partial transformation into nitric oxide. The induction of these genes is slow, as expected, as it probably needs the accumulation of toxic respiration products, such as nitric oxide, and of DNA damage, and not directly regulated by Fnr proteins. This is consistent with the fact that the nitric oxide reductase gene, *norB*, was highly induced, and its induction was very fast, reaching it maximum induction after 2 hours of anaerobic growth, suggesting that nitric oxide is the real inducer of this gene and is produced from the beginning. Both *fnrN* and *fixK* were also induced in anaerobiosis and an Fnr box was identified in the promoter of *fixK*. All the flagellar, pili and chemotaxis genes were repressed in anaerobic

Abstract

conditions, which is surprising taking into account that anaerobiosis is a non-favourable condition for TFA.

Moreover, EM has shown that TFA has a polar bunch of flagella and fimbriae, which is lost in anaerobic conditions. Three putative flagellar regulatory proteins have been identified in TFA, CtrA, FleQ and FliA, which are necessary for motility in this bacterium. TFA is the first *Sphingopyxis* strain whose flagellum has been characterised to some extent. During this characterisation, four spontaneous mutants have been isolated that swim faster than the WT strain.

Sphingopyxis granuli estirpe TFA es una α-Proteobacteria aislada del río Rin que es capaz de degradar el solvente orgánico tetralina. Hasta ahora el género *Sphingopyxis* se había descrito siempre como aerobio estricto, pero en este trabajo hemos demostrado que TFA es capaz de respirar nitrato a nitrito en condiciones anaeróbicas, convirtiéndose, por tanto, en la primera estirpe de *Sphingopyxis* anaeróbica facultativa reportada.

Se han encontrado en TFA dos posibles proteínas reguladoras anaeróbicas de tipo Fnr, FnrN y FixK, que han mostrado ser necesarias para el crecimiento anaeróbico, siendo FnrN más relevante que FixK. Los análisis transcriptómicos de esta bacteria por dRNA-seq y RT-qPCR han mostrado genes regulados diferencialmente en anaerobiosis en comparación con aerobiosis, pertenecientes a diferentes categorías funcionales. Análisis similares usando un doble mutante Δ*fnrN*Δ*fixK* han llevado a la identificación de genes directamente regulados por estos reguladores Fnr. Se ha definido un regulón de 14 operones y se ha identificado también el sitio consenso de reconocimiento de estas proteínas reguladoras, la caja Fnr. El metabolismo central de esta bacteria apenas se ve afectado in condiciones anaeróbicas, con escasas excepciones. Como se esperaba, se han inducido varias oxidasas terminales alternativas en condiciones anaeróbicas: la nitrato reductasa (*nar*), la citocromo *bd* oxidasa (*cyd*), la citocromo *o* quinol oxidasa (*cyo*), la citocromo *c* oxidasa cbb_3 (*cco*) y una oxidasa alternativa insensible a NO (*aox*). Sin

embargo, solo *nar* y *cco* mostraron sitios Fnr y parecían estar regulados por Fnr. Es interesante que muchos genes involucrados en respuesta a estrés y sistemas de reparación SOS se indujeron en condiciones anaeróbicas, sugiriendo que la anaerobiosis es un ambiente hostil y mutagénico para TFA, probablemente debido a la acumulación de nitrito como producto de respiración y su transformación parcial a óxido nítrico. La inducción de estos genes es lenta, como se esperaba, ya que necesita probablemente la acumulación de productos de respiración tóxicos, como óxido nítrico, y daño en el ADN, no estando directamente regulados por las proteínas Fnr. Esto concuerda con la alta y rápida inducción del gen de la óxido nítrico reductasa, *norB*, que alcanza su máxima inducción a las 2 horas de crecimiento anaeróbico, sugiriendo que el óxido nítrico es el verdadero inductor de este gen y que se produce desde el principio. Tanto *fnrN* como *fixK* se inducían también en anaerobiosis y se ha identificado una caja Fnr en el promotor de *fixK*. Todos los genes de flagelo, pili y quimiotaxis se reprimían en condiciones anaeróbicas, lo que es sorprendente teniendo en cuenta que la anaerobiosis es una condición no favorable para TFA.

Además, la microscopía electrónica ha mostrado que TFA tiene un penacho polar de flagelos y fimbrias, que se pierde en condiciones anaeróbicas. Se han identificado tres posibles proteínas reguladoras de flagelo en TFA, CtrA, FleQ y FliA, que son necesarias para la movilidad en esta bacteria. TFA es la primera estirpe de *Sphingopyxis* cuyo flagelo ha sido caracterizado hasta cierto punto. Durante esta caracterización, se han aislado cuatro mutantes espontáneos que nadan más rápido que la estirpe silvestre.

INTRODUCTION

1. Sphingopyxis granuli strain TFA and tetralin biodegradation

A great number of bacteria show a nutritional versatility that allow them to use multiple molecules as nutrients, a wide variety of organic pollutants produced by humans among them, some of which are recalcitrant. This makes the study of these pollutant-degrading microorganisms and the characterisation of their metabolism a very important matter in current society. The reason for this extraordinary flexibility lies not only in the variety of structural genes coded in their genomes but also, more decisively, in the way in which their regulation networks detect environmental conditions and adjust their cellular physiology to the constant changes in them.

Sphingopyxis granuli strain TFA is a Gram-negative α-Proteobacteria, belonging to the *Sphigomonadaceae* family, that was isolated from the Rhine river in Germany[1] and that is able to use the organic solvent tetralin (1,2,3,4-tetrahydronaphthalene) as unique carbon and energy source[2]. The tetralin molecule consists of an aromatic ring and an alicyclic ring that share 2 carbon atoms (Figure 1).

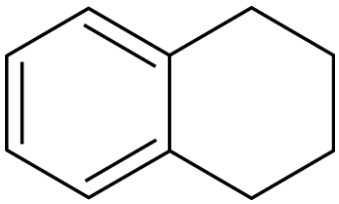


Figure 1. Tetralin molecule. This molecule consists of an aromatic ring (left) and alicyclic ring (right).

This compound of industrial interest is produced by catalytic hydrogenation of naphthalene by cracking of anthracene. Its solvent characteristics motivate its use as degreasing agent and solvent for fats, resins and waxes, as a substitute for turpentine in paints, lacquers and shoe polishes, and in connection with coal liquefaction in the petrochemical industry. Tetralin can also be found in coal tar and petroleum[3].

As a consequence of its lipophilic character, tetralin can interact with biological membranes causing changes in their structure and function, which may interfere with cell activity and growth. Although changes in membrane

composition could cause tolerance to organic solvents[4], in addition to increasing cell membrane permeability, this molecule can form hydroperoxide derivatives, highly toxic and mutagenic, inside the cells[5]. For these reasons, tetralin has been reported to be toxic for cells at concentrations higher than 110 µM (15 µL L^{-1})[6], which has also motivated its use as biocide[7].

Although tetralin biodegradation has been found and partially characterised in several bacteria[3,6,8], tetralin biodegradation pathways has only been studied and completely characterised in *Sphingopyxis granuli* TFA[9].

In TFA, the genes involved in tetralin biodegradation, the *thn* genes, are organised in four operons, M, B, H and C, expressed under the three promoters P_M, P_B and P_C respectively, and two internal promoters, P_H and P_R, located in operons B and C respectively (Figure 2A). For tetralin biodegradation, only the genes from operon C and the genes from *thnB* to *thnA2* of operon B are essential, as the function of the rest of genes can be performed by other operons in TFA involved in β-oxidation of fatty acids[9].

As shown in Figure 2B, tetralin biodegradation in TFA starts with a dioxygenation of the aromatic ring catalysed by the enzymatic complex ThnA1A2A3A4, and a subsequent dehydrogenation catalysed by ThnB, leading to the production of a re-aromatised catecholic derivative bearing two contiguous hydroxyl residues. After that, a second dioxygenation reaction catalysed by the enzyme ThnC takes place, which produces the extradiolic cleavage of the catecholic derivative in *meta* position. Later, the ThnD hydrolase breaks the C-C bond of the alicyclic ring, generating a 10-carbon long dicarboxylic acid, that after the action of the ThnE hydratase and the ThnF aldolase is transformed into pimelic acid semialdehyde and pyruvate. Pyruvate is incorporated in the central metabolism directly through the Krebs cycle, while pimelic acid semialdehyde is oxidised to pimelic acid by the aldehyde dehydrogenase ThnG. This dicarboxylic acid undergoes β-oxidation by the action of the enzymes ThnH, ThnJ, ThnK, ThnL, ThnI and ThnN, leading to the production of an acetyl-CoA, that can be incorporated into the Krebs cycle, and glutaryl-CoA, which can be further metabolised *via* crotonyl-CoA to form two acetyl-CoA molecules[9].

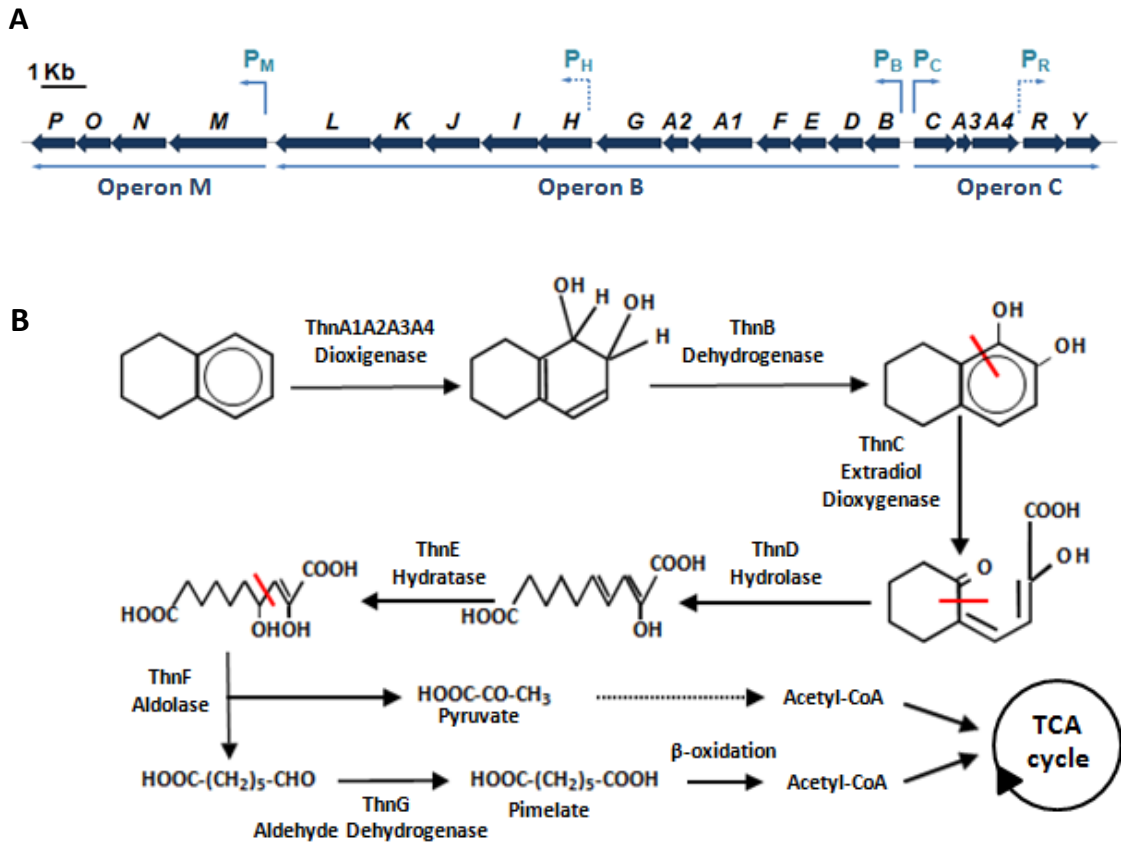


Figure 2. Tetralin biodegradation in *Sphingopyxis granuli* TFA. (A) Genetic organisation of the *thn* genes. (B) Tetralin biodegradation pathway.

Regarding the regulation of these operons, tetralin biodegradation genes are only induced in the presence of tetralin and in the absence of a preferential carbon source. Three regulatory proteins are involved in this induction of *thn* genes: the Lys-R type regulator ThnR, the ferredoxin like reductase ThnY and the ferredoxin ThnA3. ThnR regulator and its co-activator ThnY, present in basal levels provided by the weak constitutive promoter P_R, are essential for the expression of *thn* genes. The ferredoxin ThnA3 can deliver the electrons to the dioxygenase ThnA1-ThnA2 or to ThnY. In the presence of tetralin, ThnA3 delivers the electrons preferentially to ThnA1-ThnA2, which catalyses the first dioxygenation of the tetralin molecule. Under these conditions, ThnR and the oxidised form of ThnY are able to activate the *thn* promoters, leading to tetralin biodegradation. In the presence of compounds that are not substrates of the dioxygenase, ThnA3 transfers the electrons to ThnY, which accumulates in its reduced form, which is unable to co-activate the expression of *thn* genes together with ThnR[9].

In the latest years, the complete genome of TFA has been sequenced and annotated[10] and, besides the genes for tetralin biodegradation, additional important information was extracted from the genes found in this annotation. One of them was the existence of genes putatively involved in anaerobic respiration. This was interesting because *Sphingopyxis* genus had been described in 2001 as a group of strictly aerobic bacteria[11], therefore, this finding could make TFA the first facultative anaerobic *Sphingopyxis* strain reported[10].

2. Aerobic and anaerobic respiration

Respiration is a biological process whose purpose is to generate energy for the cells through oxidative phosphorylation, consisting of the transfer of electrons from reduced substrates with low redox potential (*e.g.* NADH, carbonated substrates) to final electron acceptors. This transference takes place through redox cofactors, and the final electron acceptor can be oxygen, in the case of aerobic respiration, but there are alternative final electron acceptors that can be used by bacteria, which are the basis of anaerobic respiration. The energy liberated in this electron transfers is used to translocate protons though the bacterial membrane from the cytoplasm to the extracellular space or the periplasm, creating a electrochemical gradient of protons, proton motive force (PMF), which can be used to generate ATP.

Electrons liberated from the substrates are transferred to quinones in the cytoplasmic membrane, reducing them to quinols. Then, electrons can be transferred to two different kinds of final oxidases: cytochrome c oxidases or quinol oxidases, that reduce O_2 to H_2O, or alternative oxidases that use final electron acceptors different from O_2, such as nitrate, nitrite, fumarate, DMSO, etc (Figure 3).

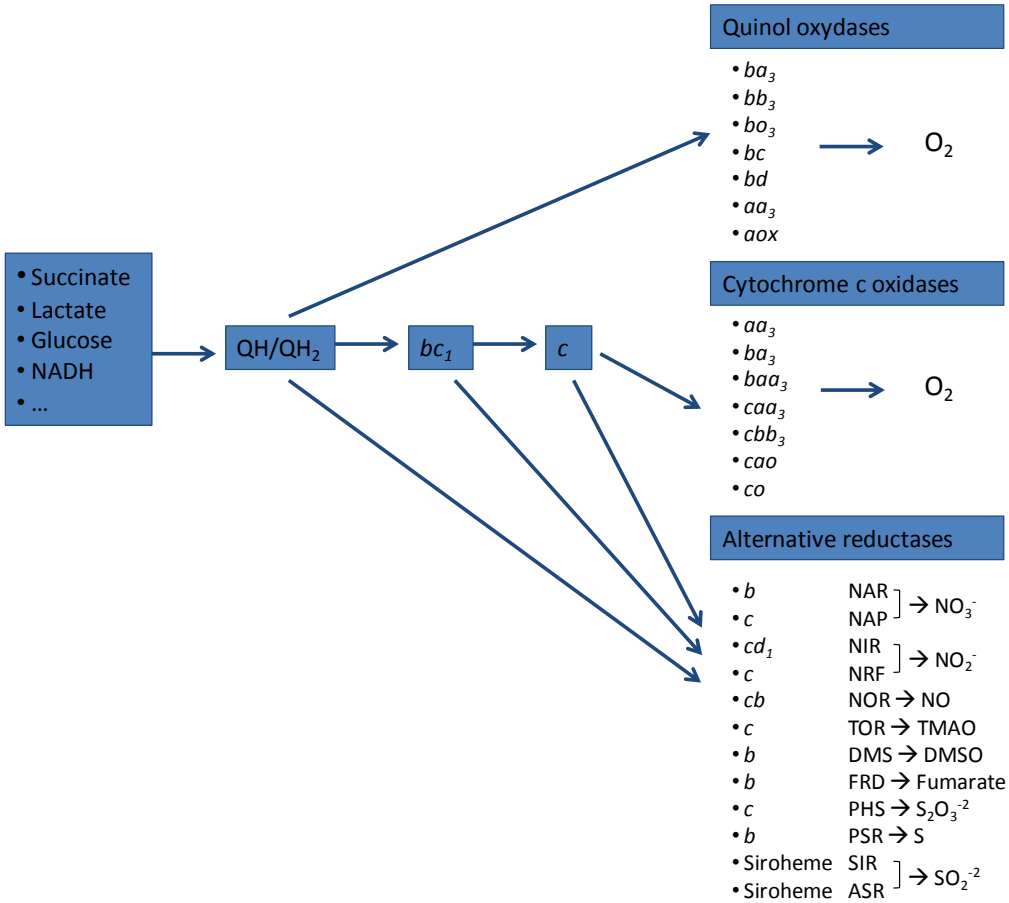


Figure 3. Bacterial respiratory chains. Electrons from carbonated substrates (succinate, lactate, glucose, etc.) or reduced cofactors (NADH, $FADH_2$) are transferred to quinones (QH) reducing them to quinols (QH_2) and then to different types of final oxidases: quinol and cytochrome c oxidases for aerobic respiration and alternative oxidases for anaerobic respiration. Figure adapted from a previous work[12].

In contrast to eukaryotic mitochondrial respiratory system, bacteria show branched respiratory chains with multiple final oxidases, each one with different affinity for their final electron acceptor[13].

2.1. Aerobic and microaerobic respiration

Focusing on oxygen respiration, two types of oxygen reductases have been described depending on the substrates used as electron donor: cytochrome *c* oxidases, that receive electrons from reduced c-type cytochromes, and quinol oxidases, that receive electrons from hydroquinones. Cytochrome *c* oxidases are more energetically efficient, as they generate a higher H^+/e^- rate, thus generating more ATP[14]. Regarding quinol oxidases, it was proposed that they

have evolved from cytochrome oxidases by loss of the Cu_A centre, as this loss could be reverted by mutating a few residues, as it was done for *E. coli* bo_3 quinol oxidase[15]. Other authors proposed a different idea, taking into account that sequence analyses are only valid if functional homology is observed[16]. They suggest that that cytochrome *c* and quinol oxidases translocation is not achieved by the same mechanism: for cytochrome *c* oxidase they proposed a proton pumping mechanism with the involvement of Cu_A, while quinol oxidases would make use of a Q-cycle.

Oxygen reductases can be classified in three families based on sequence homology, function, reduction sites and affinity for oxygen: Heme-copper oxidases (HCOs), Alternative oxidases (AOX) and cytochrome bd oxidases[17].

2.1.1. Heme-copper (HCOs) family

This family is characterised for having an membrane integral subunit I that carries as cofactor a low-spin heme and a high spin heme-copper binuclear centre (Cu_B site), composed by a high spin heme (a_3, o_3 or b_3) and a copper ion, where O_2 is reduced to H_2O. HCOs, moreover, produce the PMF using a "proton-pump" mechanism. This family includes both quinol oxidases and cytochrome *c* oxidases[14,17].

Most HCOs belong to one of these 3 subfamilies[18,19]: type A (oxidases aa_3) that are structurally and functionally close to those of the mitochondria, type B (cytochrome oxidases ba_3), and type C (cytochrome *c* oxidases cbb_3) (Figure 4). Type A oxidases include the mitochondrial cytochrome *c* oxidases and most prokaryotic cytochrome *c* oxidases and quinol oxidases, whilst type B oxidases include oxygen reductases from extremophilic prokaryotes[17]. Type C comprises around 20% of HCOs and they are essential for the pathogenenicity of many bacteria[20].

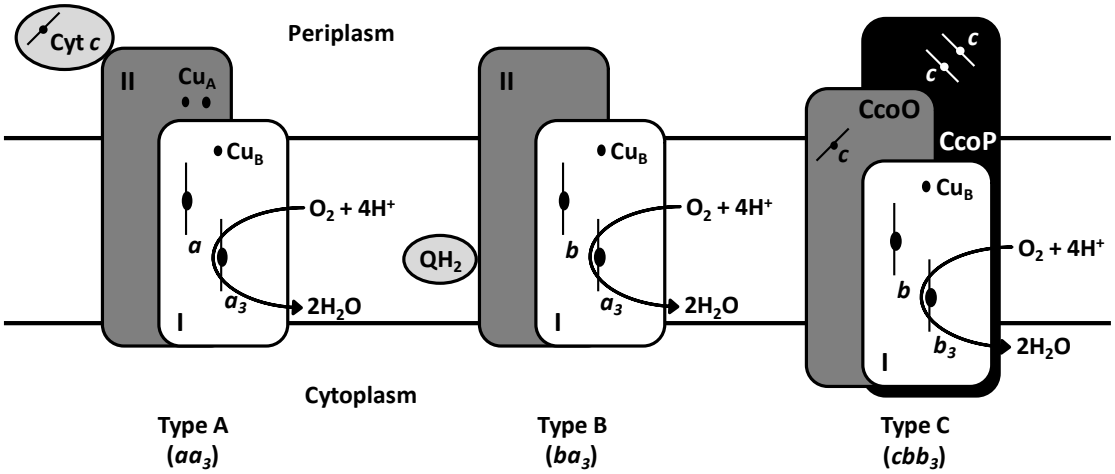


Figure 4. Types of heme-copper oxidases. The three different types of heme-copper oxidases with their multiple subunits are represented here: type A (aa_3 oxidases), B (ba_3 oxidases) and C (cbb_3 oxidases). Adapted from a previous work[14].

In addition to subunit I, the HCOs are composed of at least another subunit, named subunit II in types A and B and CcoO or FixO in type C. While quinol oxidases have a cofactor-free subunit II, cytochrome *c* oxidases have cofactors bound to this subunit, most frequently a binuclear Cu-Cu centre (Cu_A site) liganded by six highly conserved aminoacids (HisII-181, CysII-216, GluII-218, CysII-220, HisII-224 and MetII-227)[18,21]. Subunit III in types A and B and the non-conserved subunit IV of HCOs are cofactor-free[18]. The variety of electron donors they can use, their subunit composition and the type of heme groups that they contain make HCO family a very versatile family of oxidases.

In microoxic conditions, where oxygen concentrations are low, bacteria induce the expression of oxidases with high affinity to oxygen, in order to be able to respire at these low concentrations. The cbb_3 cytochrome *c* oxidases show high affinity to oxygen, allowing respiration under microoxic conditions in *Bradyrhizobium japonicum*[22,23]. Genes encoding for the subunits of the cbb_3 complex were first isolated from rhizobia and called *fixNOQP* as they were required for symbiotic nitrogen fixation[23]. However, the orthologous genes *ccoNOQP* were found in other Proteobacteria, some pathogenic bacteria among them, suggesting that these genes were also needed in human pathogens to colonise anoxic tissues[14]. As said, the multiple subunits of this cbb_3 oxidase are encoded in *ccoNOQP* operon, but its biogenesis depends also

on the expression of *ccoGHIS* operon, which products are thought to be responsible for cofactor insertion and maturation of cbb_3 enzymes.

The regulation of *ccoNOQP* is different depending on the organism, but it has been observed to be regulated by Fnr in *Rhodobacter sphaeroides*, *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *Rhizobium leguminosarum* and *R. capsulatus*, by FixLJ/FixK in *Sinorhizobium meliloti*, *B. japonicum*, *Azorhizobium caulinodans* and other rhizobial species and even to be expressed constitutively in *Helicobacter pylori* and *Campylobacter jejuni*[14].

Another HCO is the cytochrome *o* quinol oxidase encoded by *cyoABCDE* operon. It has been reported in some bacteria like *E. coli* and *P. putida* that *cyoA*, *cyoB*, *cyoC* and *cyoD* code for the subunits II, I, III and IV of the cytochrome *o* oxidase, while *cyoE* encodes a heme *o* synthase[24,25]. This oxidase has two protoheme IX groups (cytochromes b_{555} and b_{562}) and two copper atoms per complex[26,27]. Cyo oxidase has been reported to have low affinity for oxygen[28] and in *E. coli* it is used when oxygen concentration is high[29]. However, in *Rhizobium etli* Cyo is used during the early development of its symbiosis with *Phaseolus vulgaris* and is important to adapt to and maintain growth in low oxygen conditions[30].

TFA has shown both a *ccoNOQPGHIS* operon and a *cyoABCD* operon in its sequence annotation.

2.1.2. Alternative oxidase (AOX) family

The alternative oxidase family comprises cyanide and NO-resistant oxidases found in prokaryotes and in the mitochondria of higher plants, fungi, protists and some animal species[31,32]. These oxidases use UQH_2 as electron donor, but not cytochrome *c*, and their active site for O_2 reduction consist on a non-heme di-iron carboxylate site. They do neither produce PMF nor are they coupled to transmembrane charge transfer, but, although their phisiological role is still not very clear, they seem to play an important role in energy metabolism, facilitating turnover of the TCA cycle, protection against oxidative stress, and growth homeostasis[17]. Additionally, thanks to its resistance to NO, it has been reported in *Vibrio fischeri* to be transcriptionally activated in the presence of nitric oxide

by the NO-responsive regulator protein NsrR[33] and to help reduced the stress caused by NO[34]. One gene putatively coding for an Aox has been found in TFA sequence.

2.1.3. Cytochrome *bd* family

The family of cytochrome *bd* oxidases is exclusively integrated by quinol oxidases, most commonly using ubiquinol and menaquinol as substrates. Because of their high affinity for oxygen, they are induced in low oxygen conditions in many organisms. These oxidases show no sequence homology to any subunit of heme-copper family members or to the alternative oxidases and do not contain any copper or non-heme iron in the catalytic centre. They are two-subunit integral membrane proteins with three hemes, b_{558}, b_{595} and *d*, being hemes b_{595} and *d* probably forming a di-heme site for O_2 reduction. Cytochrome *bd* oxidases also generate PMF by transmembrane charge separation, but they do not make use of "proton pump" for this purpose, having thus a lower total energetic efficiency compared to heme-copper type oxidases[14,17]. These oxidases are only found in bacteria and archaea, in contrast to heme-copper enzymes that are found in many other organisms[35].

From this family, the best studied representative is cytochrome *bd*-I from *E. coli*, a two-subunits complex encoded by the operon *cydAB*[36]. These subunits carry three hemes, heme b_{558} located in subunit I (CydA) and hemes b_{595} and *d* likely located in the area of the subunit contact[37-39]. Additionally, the operon *cydCD*, codes for two proteins, CydC and CydD, that are essential for the assembly of *bd*-I, although they are not subunits of this oxidase[40-43]. In fact, this operon encodes a heterodimeric ATP-binding cassette-type transporter that is glutathione transport system[44]. The operons *cydAB* and *cydDC* have also been found in TFA.

2.2. Alternative final electron acceptors for anaerobic respiration and their respiratory chains

As we have mentioned previously, oxygen is the final electron acceptor for respiration used in the mitochondria of eukaryotic organisms, as well as in many prokaryotic organism, in order to obtain energy. However, bacteria are also able

to use alternative final electron acceptor for respiration, which give them a biological advantage, contributing to their ability to colonise and sustain life in many microoxic and anoxic environments, uninhabitable for strict aerobic organisms. Thus, anaerobic respiration is a form of respiration in which the microorganisms use final electron acceptors different from oxygen, such as nitrate, nitrite, nitric oxide, nitrous oxide, dimethyl sulphoxide (DMSO), sulphate, sulphite, fumarate or even metals like Fe^{3+}, needing the presence of certain respiratory enzymes to catalyse these reactions. Here we are going to focus on nitrate anaerobic respiration, which is the aim of study of this thesis.

2.2.1. Nitrate respiration and denitrification

Nitrate reductases can be classified in 4 major groups: eukNR, Nas, Nap and Nar[45]. The eukNR are the nitrate reductase present in eukaryotic organisms that perform nitrate assimilation in plants and fungi. The other three nitrate reductases are prokaryotic and can reduce nitrate in an assimilatory (Nas) or dissimilatory (Nar and Nap) process[45]. In assimilatory reduction, an anabolic pathway, nitrate is first reduced to nitrite by nitrate reductase and subsequently transformed to ammonia by assimilatory nitrite reductases and incorporated in the organism's biomass. On the other hand, dissimilatory reduction of nitrate is a catabolic process, where the products of the reduction process are not incorporated in the cell but excreted. As for their cellular location, the assimilatory nitrate reductases (eukNR and Nas) are soluble cytoplasmic proteins, while the dissimilatory nitrate reductases (Nap and Nar) are membrane associated. However, the Nap complex is associated with the periplasmic side of the membrane, while de Nar complex faces the cytoplasm in bacteria and the periplasm in Archaea (pNar)[46,47]. Nar reductases are generally associated to nitrate anaerobic respiration for energy production. These complexes couple nitrate respiration with proton translocation across the membrane, generating a PMF that can be used to generate ATP by an ATP-synthase. On the other hand, Nap reductases, although they can be used for nitrate respiration, are predicted to produce less ATP than Nar reductases, as both the electron donor and acceptor complexes are oriented into the periplasm and no proton gradient should be formed. However, when nitrate reduction can be coupled to energy conservation if a NADH dehydrogenase or a formate

dehydrogenase forms the proton gradient, though this gradiente is smaller than with Nar[48]. This loss of potential gradient could be compensated, however, with the higher affinity for nitrate that these Nap generally show[49]. Nap reductases have also been associated to other diverse functions, such as the cellular oxidation-reduction potential maintenance[13] and nitrate removal[50-52].

Focusing on nitrate dissimilatory reduction, nitrate is one of the most commonly used final electron acceptors for anaerobic respiration. Some bacteria like *E. coli* are able to respire nitrate to nitrite and then to ammonia[53], other bacteria are only able to respire nitrate to nitrite and other, like the rhizobia, are able to transform it to molecular nitrogen in a pathway called denitrification[54] (Figure 5).

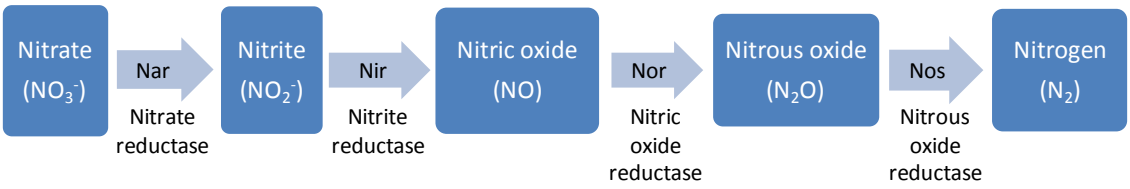


Figure 5. Denitrification pathway. Here the complete denitrification pathway is shown, indicating the enzymes that perform each reaction.

Nitrate respiration mechanisms have been well characterised in several bacteria like *E. coli*[55], *P. denitrificans*[13], *Rhodobacter sp.*[56] and *P. aeruginosa*[57]. In Figure 6, Nar proteins and *nar* genes of *P. aeruginosa* are shown. NarXL is the a component system that senses nitrate in this bacteria, consisting of the sensor kinase NarX that in the presence of nitrate phosphorylates its response regulator NarL. This NarL regulator is essential for the activation of the *narK$_1$K$_2$GHIJ* operon expression[58], which codes for the nitrate/nitrite antiporter NarK$_1$K$_2$ and the three components of the nitrate reductase NarGHI[59,60]. NarJ is not a component of the final enzyme complex but it is important for its formation[61]. Reduction of nitrate by the nitrate reductase is coupled to quinol oxidation and consumes protons from the cytoplasm, that are then transferred to the periplasm, contributing to the creation of a proton gradient and, therefore, a PMF[62].

Introduction

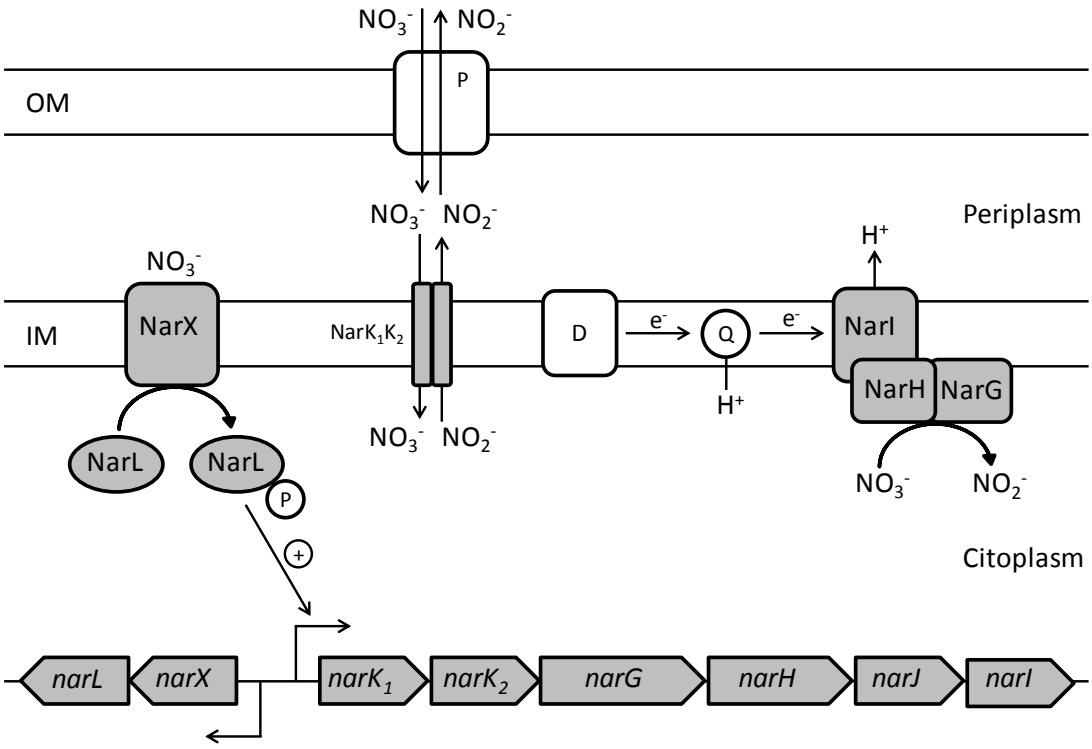


Figure 6. Nar proteins, *nar* genes and nitrate respiration in *P. aeruginosa*. Image shows the *nar* genes of *P. aeruginosa*, as well as the Nar proteins (both coloured in grey): the two-component nitrate-sensing system NarXL, the nitrate/nitrate antiporter $NarK_1K_2$ and the three subunits of the nitrate reductase NarGHI. "P" is an unknown porine for nitrite and nitrate transfer through the outer membrane; "D" stands for several dehydrogenases for example for NADH oxidation, etc.; "Q" refers to the ubiquinone/ubiquinol pool. Figure adapted from a previous work[57].

Some bacteria, like *E. coli*, have the $NarK_1K_2$ antiporter fused in one single protein, called NarK or NarU. In addition, regulation may also be different in other bacteria. For instance, α-Proteobacteria have a different regulator for *narK₁K₂GHIJ* operon, namely NarR[63], instead of the *narXL* operon of *P. aeruginosa*.

The preliminary sequence analysis of TFA has shown that this bacteria has in its genome the *narUGHJI* operon as well as a gene homologous to the *narR* gene, *ftrB*, being likely able to respire nitrate to nitrite (Figure 7).

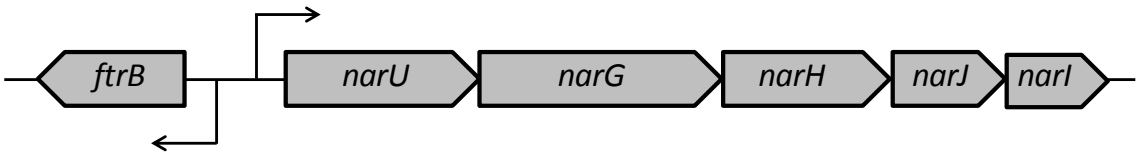


Figure 7. Nitrate reductase *nar* genes in TFA. Figure shows the *nar* genes of TFA: the nitrate/nitrate antiporter gene *narU*, the genes coding for the subunits of the nitrate reductase *narGHI*, the gene coding for *narJ* and the gene coding for the regulator *ftrB* (homologous to *narR*).

Regarding nitrite respiration, in denitrifying bacteria two different nitrite reductases have been described: NirS and NirK. NirS is a homodimeric enzyme with hemes *c* and d_1. The *nirS* gene is part of a gene cluster, being this cluster best characterised in the denitrifying species *P. aeruginosa* (*nirSMCFDLGHJEN*), *P. denitrificans* (*nirXISECFDLGHJN*) and *P. stutzeri* (*nirSTBMCFDLGH* and *nirJEN*). On the other hand, NirK is a copper-containing enzyme, a homotrimeric complex with three type I and three type II copper centres forming the active site. Nitrite binds to the copper ion in the type II centre replacing the exogenous ligand, that can be water or chloride, and nitrite is reduced to nitric oxide by electron transfer from the type I copper site. Both NirS and NirK proteins are located in the periplasm, and they receive the electrons from cytochrome *c* and/or the blue copper protein pseudoazurin through the cytochrome bc_1 complex, catalyzing a one-electron reduction of nitrite to nitric oxide[14].

Regarding the next step of the denitrification process, the respiration of nitric oxide to nitrous oxide, three different types of nitric oxide reductases have been described: cNor, qNor and qCu_ANor[14]. The best studied group is cNor, which use as electron donors soluble or membrane c-type cytochromes or small soluble blue copper proteins (azurin, pseudoazurin)[64]. This nitric oxide reductase, which has been isolated from *P. stutzeri* and other Proteobacteria[65,66] is encoded by the operon *norCBQD*. The nitric oxide enzyme is a complex form by two proteins, NorB and NorC. NorC is a membrane-anchored *c*-type cytochrome, while NorB has two *b*-type hemes and a non-heme iron. Electrons from donor molecules are transferred to the heme *c* of NorC and then to the active site through the heme *b* of NorB, subsequently

reducing two molecules of nitric oxide to nitrous oxide and water[14,67]. Although these proteins are closely related to HCO, they are not proton pumps or electrogenic[68]. On the other hand, *norQ* and *norD* genes of the operon encode for proteins essential for the activation of the nitrate reductase. Other more specialised denitrifiers have other proteins involved in maturation and/or stability of Nor activity, encoded by the *norEF* genes[69].

The nitric oxide reductases of the second type, the qNor reductases, do not consist of two subunits as the cNor reductases, but of one longer one, a NorB protein with an N-terminal extension including two additional transmembrane regions flanking a putative periplasmic domain[70,71]. Figure 8 shows the structural differences between cNor and qNor reductases. Moreover, these enzymes use quinol or menaquinol as electron donors and they are not only found in denitrifying archaea and soil bacteria, but also in non-denitrifying pathogenic bacteria, where it is used for detoxification purposes[14,67].

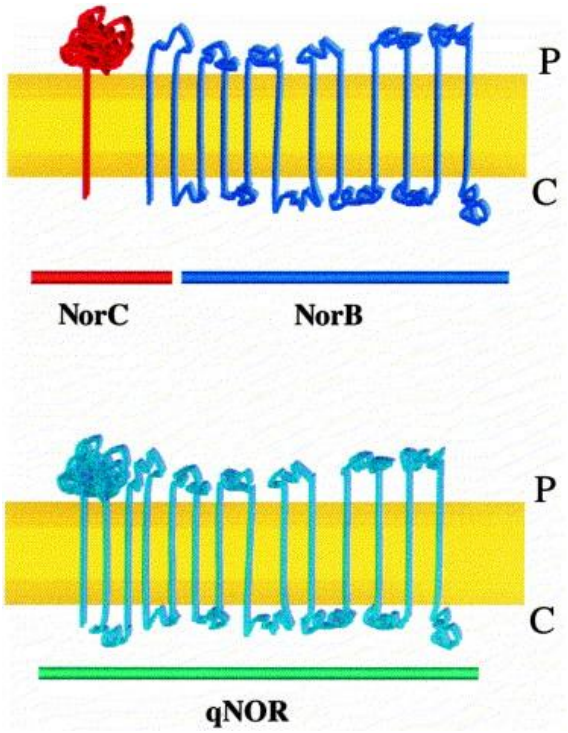


Figure 8. Structure comparison between cNor and qNor. Figure shows the predicted topology of the multi-subunits cNor (above) and the single-subunit qNor (underneath), with their respective gene represented under each predicted image. P: periplasm. C: for the cytoplasm[67].

Finally, the last type of Nor, the qCu_ANor has only been found until now in the Gram-positive bacteria *Bacillus azotoformans*[72]. This enzyme is able to use both menahydroquinone (MKH_2) and a specific *c*-type cytochrome c_{551} as electron donors. One hypothesis is that the use of MKH_2 is linked to detoxification purposes while the use of c_{551} with a bioenergetic function[14]. Our preliminary sequence analyses of TFA suggest that this bacteria encodes for a qNOR enzyme, probably involved in NO detoxification.

As for the last step of the denitrification pathway, the respiration of nitrous oxide to dinitrogen (N_2), a two-electron reduction is carried out by the nitrous oxide reductase (Nos) enzyme. This reductase is a homodimer of two copper-containing subunits, each of which has two closely located domains: the "Cu_A" domain, where nitrous oxide binds, and the "Cu_Z" domain, which receives the electrons donated by *c*-type cytochromes or cupredoxins[73-75]. The nitrous oxide reductase is generally encoded within the *nosRZDFYLX* operon, *nosZ* being the gene encoding for the monomers of the enzyme. It has been suggested that the *nosDFYL* genes are necessary for copper assembly into the nitrous oxide reductase, but their specific role is still unknown. Furthermore, the NosRX proteins are involved in transcription regulation, activation and Cu assemblage of Nos[76]. This reductase has been isolated from a great number of denitrifying strains *P. denitrificans*, *P. pantotropus* and *P. stutzeri* among them[14].

2.3. Oxygen sensing and anaerobiosis regulatory proteins

Regarding global regulation in anaerobic conditions, to date, three main modes to sense oxygen have been described in bacteria. Two of them involve the direct interaction of oxygen with a protein, that can be either a membrane protein receptor, as in the FixL sensor quinase in rhizobia, or a cytoplasmic transcriptional factor, *e.g.* Fnr in *E. coli* and many other bacteria. The third method is based on monitorising environmental oxygen concentration by detecting changes in the redox state of molecules or pools of molecules *via* different sensor proteins[14].

A group of bacteria commonly called "the rhizobia", which comprises members of several genera of the Rhizobiaceae family, are able to live both in soil and laboratory culture (free-living) as well as endo-symbiotically inside infected cells

of legume root nodules. The bacteria in this symbiotic state, called bacteroids, are able to fix molecular nitrogen, using it as nitrogen source. The low oxygen conditions this bacteria find in the root nodules induce the expression of specific genes in order to adapt to these conditions[77]. $FixK_2$ is a transcriptional factor belonging to the cyclic AMP (cAMP) receptor protein (Crp) superfamily of transcription factors that trigger physiological changes in response to a variety of metabolic and environmental cues. $FixK_2$ is able to recognise specific sequences in the genome, the Fnr boxes, regulating the expression of genes that need to be differentially regulated in anaerobic conditions. However, $FixK_2$ lacks Fe-S clusters, thus it is unable to directly sense oxygen concentration. For that reason, it is the two-component regulatory system the one in charge of detecting low oxygen concentrations and induce the expression of *fixK*$_2$ in response[14]. A moderate decrease of oxygen concentration of 5% in the gas phase triggers ATP-dependent autophosporylation of FixL hemeprotein and the later transference of this phosphoryl group to the response regulator FixJ[78,79] (Figure 9). $FixK_2$ plays a dual role as it inhibits its own expression in some organisms as *B. japonicum* and activates the genes involved in adaptation to microoxia, such as the *fixNOQP* genes that allow bacteroid respiration inside root nodules[80,81].

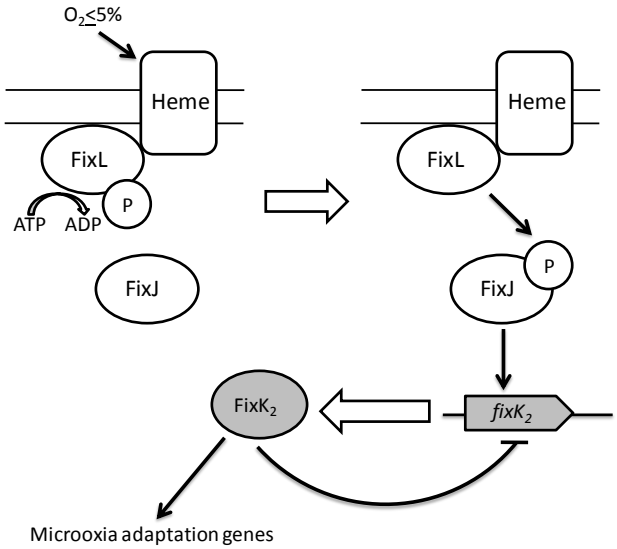


Figure 9. FixK-FixLJ system in rhizobia. Low oxygen induced the autophosphorilation of FixL and the subsequent transfer of the phosphoryl group to FixJ, which activates the expression of *fixK*$_2$. $FixK_2$ inhibits its own expression and induced the expression of genes involved in adaptation to microoxia.

Regarding the redox state mechanism to sense oxygen limitation, there are different protein system depending on the organism: ArcBA system in *E. coli*, RegBA/PrrBA systems in *Rhodobacter* ssp., RoxSR system in *Pseudomonas* ssp., RegSR system in *B. japonicum* and ResDE and Rex systems in *Bacilus subtilis*[14]. Similarly to FixLJ system, *E. coli* ArcBA is a two-component system in which the tripartite membrane-bound sensor kinase ArcB is autophosphorylated under low oxygen conditions and subsequently transfer this phosphoryl group to the response regulator ArcA[82]. Phosphorylated ArcA is able to activate the expression of genes and/or operons involved in adaptation to low oxygen conditions, *e.g.* high oxygen affinity oxidases, and to repress the expression of aerobic oxidases[83]. However, ArcB is not able to directly sense oxygen concentration, but it is regulated by the redox state of the UQ-ubiquinol pool in the aerobic respiration chain, as UQ inhibits ArcB autophosphorylation, and also by the redox state of the MK pool[84,85].

2.3.1. Fnr proteins

The oxygen sensing protein Fnr, also belongs to the Crp superfamily of transcriptional factors. These proteins consist of 4 functionally different domains: an oxygen-sensing domain, a series of β strands (β-roll structure) in a loop-like structure that make contact with the RNA polymerase, a long α-helix that acts as a dimerisation interphase and a helix-turn-helix motif in the C-terminal involved in DNA binding[14]. Comparing the sequences of *fnr* genes of different bacteria, four amino-proximal cysteins of the ligand-binding region, corresponding to cysteins 20, 23, 29 and 122 of *E. coli* Fnr, have been identified to be highly conserved. Fnr members belonging to the FnrN group, present a displacement of 2 positions to the right in the 3rd highly conserved cystein with respect to that of *E. coli*[86]. These 4 Cys residues are able to bind either a [4Fe-4S]$^{2+}$ or a [2Fe-2S]$^{2+}$ cluster[87,88]. Additionally, three amino acid residues of the second helix of the helix-turn-helix motif of the DNA binding region, a glutamic acid, a serine and an arginine (E-209, S-212 and R-213 in *E. coli* Fnr) are also reported to be highly conserved in Fnr proteins[88,89].

The *fnr* gene is essential in *E. coli* and many other bacteria for the expression of genes involved in anaerobic metabolism in the absence of oxygen. In *E. coli*,

Introduction

Fnr is reported to transcribe 63 target genes[90]. A conserved palindromic region, the Fnr box, is located upstream of the genes and operons regulated by Fnr. When oxygen concentration becomes limiting, Fnr acquires a $[4Fe\text{-}4S]^{2+}$ cluster, undergoing a structural change at the dimerisation interphase[88]. This structural change causes Fnr to dimerise, going from being an inactive monomer to being an active dimer that binds to its target sequences, the Fnr boxes[91,92]. Bound at its sites, Fnr activates the expression of the target genes by recruiting the RNA polymerase or inhibits it by preventing the effective binding of the RNA polymerase to the DNA[87]. In the presence of O_2, the $[4Fe\text{-}4S]^{2+}$ cluster interacts with this O_2 and is converted to $[2Fe\text{-}2S]^{2+}$, leading to loss of DNA binding and monomerisation of Fnr[92]. This cluster conversion happens in two steps. The first step involves a one-electron oxidation of the cluster, releasing Fe^{2+} and generating a $[3Fe\text{-}4S]^{1+}$ intermediate and a superoxide ion. In the second steps, the intermediate rearranges spontaneously forming a $[2Fe\text{-}2S]^{2+}$ cluster and releasing a Fe^{3+} ion and two sulphide ions[87].

Two basic types of Fnr-induced promoters have been described, namely Class I and Class II promoters. Class I promoters have the FNR sites located close to -61.5, -71.5, -82.5 or -92.5 with respect to the transcription initiation site, allowing the formation of just one protein-protein contact, between Fnr and the C-terminus of the RNA polymerase α-subunit[93,94]. Class II promoters, the most common ones, have the Fnr site centred at or close to -41.5 with respect to the transcription initiation site, which permits the formation of multiple protein-protein contacts between RNA polymerase and Fnr[94]. In the case of promoters repressed by Fnr, there are more than one binding site for Fnr in the region, *e.g.* in *E. coli*, positions -50.5 and -94.5 with respect to the transcription initiation site for the *ndh* promoter[95], positions -75.5 and -106.5 and +107.5 for the *narX* promoter[96], and +0.5 (overlapping the transcription start point) and -103.5 for the *fnr* promoter[96,97]. Fnr can repress expression by binding the promoter region in a way that blocks RNA polymerase from binding to important promoter elements (promoter occlusion). However, the fact that the upstream DNA site of Fnr is essential, suggests that repression can take place by a more complex mechanism[98].

3. Stress response and detoxification

During anaerobic respiration of nitrate, as we have seen, some respiration products are generated, namely, nitrite, nitric oxide, nitrous oxide and molecular nitrogen, depending on the organism. The accumulation of some of these products, however, has been reported to generate a stress situation for some bacteria.

Nitrite has been shown to be toxic both in aerobic and anaerobic conditions in different bacteria. In *P. aeruginosa*, nitrite substantially inhibits glucose and proline transport at concentrations of 10 mM and inhibits it completely at concentrations higher than 25 mM. This process takes place because of the oxidation of electron transfer proteins such as cytochrome oxidases, inhibiting ATP generation and active transport of glucose and proline[99]. Nitrite was also reported to inhibit active transport, oxygen uptake and oxidative phosphorylation in aerobic bacteria, as well as aldolase in *E. coli*, *P. aeruginosa* and *Streptococcus faecalis*[100]. In anaerobic conditions, nitrite accumulation has been reported to inhibit nitrate respiration in *P. denitrificans* because of the oxidation of the components of the respiratory chain[101].

Nitric oxide has been well documented to cause nitrosative stress, though the precise mechanism to sense this stress and respond to it are still to be determined more clearly. In anaerobic conditions NO is normally produced by respiration of nitrite by the nitrite reductase, though the enteric nitrate reductase NarG has been reported to also produce NO during anaerobic nitrate respiration[102,103]. Dinitrogen trioxide has also been reported as mutagenic. This compound can be generated from $NO^{\bullet}$ in the presence of oxygen or by condensation of two molecules of nitrous acid in anaerobiosis[104].

On the other hand, the activation of the SOS response in anaerobic conditions is not usual, but it has been reported in *Corynebacterium glutamicum*, which reduces nitrate to nitrite, accumulating it[105], and *Neisseria gonorrhoeae*, that uses nitrite as final electron acceptor[106]. Moreover, it is interesting that many genes that are affected in anaerobic conditions in *N. gonorreae* have also been reported to respond to iron and/or oxidative stress[106].

In anaerobiosis, adaptation to stress conditions has also been also reported to involve ribosomes modifications. RlmH, whose gene is induced in anaerobic conditions in *E. coli*, methylates the pseudouridine 1915 of the 23 S rRNA that seems to provide fitness advantage under stress conditions[107].

3.1. Oxidative stress

Reactive oxygen species (ROS) are highly reactive molecules, because of the presence of impaired electrons in their external orbitals, that can damage macromolecules such as proteins, lipids and nucleic acids. ROS can be produced from oxygen, in the presence of redox active moieties, where this O_2 can be reduced to superoxide ($O_2^{\bullet-}$) or hydrogen peroxide[108]. Moreover, hydrogen peroxide can react with Fe^{2+} through Fenton reaction generating the hydroxyl radical ($^{\bullet}OH$)[109]. ROS can also be produced by intracellular enzymatic reactions such as those mediated by aspartate oxidase and xanthine oxidase[110,111]. Moreover, the presence of metal or metalloids[112-114] or organic pollutants such as polychlorinated benzenes[115], quaternary ammonium compounds[116], hypochlorite[117] and the degradation of aromatic hydrocarbons[118] can trigger the generation of ROS.

There are two main strategies to battle with oxidative stress and ROS, the non-enzymatic and the enzymatic ways. The non-enzymatic ways involves the use of small molecules as a defence against oxidative stress, glutathione and thioredoxin being the best characterised in bacteria. Glutathione (GSH) is a reduced tripeptide of Glu-Cys-Gly that can be oxidised by an oxidant, for example a ROS, generating a cystein bridge between two GSH and generating one GSSG molecule. This reaction can be catalysed by the glutaredoxin or the GSH peroxidase enzymes and reversed by GSH reductase and thioredoxin. On the other hand, thioredoxin is a small redox protein that has two neighbouring CXXC motifs that can be oxidised by an oxidasing agent or by thioredoxin peroxidase, going from 2SH to S-S. The activity of both GSH reductase and thioredoxin reductase requires the use of cofactor NADPH to perform the reduction. These enzymes act as a link between the non-enzymatic and the enzymatic way of ROS depletion[119].

Regarding the enzymatic way, there are also several enzymes that are involved in the removal of the different types of ROS. The superoxide in unable to cross the cell membrane, so it is transformed to hydrogen peroxide or molecular oxygen by cytoplasmic and periplasmic isoenzymes of superoxide dismutase (SOD). In *E. coli* these reactions are performed by iron- and manganese-dependent or copper- to zinc- dependent SOD respectively[120]. For hydrogen peroxide depletion, catalase and alkyl hydroperoxide reductase (peroxiredoxin) are used, being these enzymes encoded in *E. coli* by *katG*, *katE* and *ahpCF* respectively[121]. Catalase make use of a heme in order to reduce peroxide to water and molecular oxygen, while alkyl hydroperoxide reductase use FAD and NAD(P)H for this purpose. Catalases have shown to be more efficient to reduce high concentrations of peroxide as they do not need the use of a reducing equivalent. However, under low stress conditions, alkyl hydroperoxide reductases seem to be the favourite option as they do not involve a dangerous ferryl/radical intermediate[120]. Peroxiredoxins are a large family of proteins divided in 6 sub-groups with distinctive amino acid sequences, but all of them containing the thioredoxin fold and the PXXT(S)XXC motif. The different peroxiredoxins can use 2-Cys or 1-Cys mechanisms, depending on the number of Cys residues involved in catalysis[122].

To date no enzymes have described to be able to reduce the hydroxyl radical, which is thought to be only removed by the non-enzymatic mechanism[119].

3.2. Nitric oxide response

Similarly to ROS, reactive nitrogen species (RNS) are molecules whose impaired electrons in their external orbitals make them highly reactive, and very prone to cause damage to cell macromolecules. The production of RNS depends on the redox state of the environment and is also mediated by the presence of ROS[123].

Nitric oxide ($^{\bullet}NO$) is considered to be the primary RNS, that can cause the generation of other RNS by reaction with ROS, particularly to peroxynitrite ($ONOO^-$), by biradical reaction between nitric oxide and superoxide[123,124]. NO can also interact with oxygen and generate the nitrosating agent dinitrogen

trioxide (N_2O_3)[103]. Moreover, NO is a labile molecule that can cross the cytoplasmic membrane[125].

In *E. coli* the main transcriptional regulators involved in sensing the presence of NO and regulate the expression of genes in response to it are NorR and NsrR[126,127]. NorR is an enhancer protein necessary for the expression of the genes of the NO reductase, flavorubredoxin and flavorubredoxin reductase, *norV* and *norW,* and it has not been found to regulate any other genes in *E. coli*[103,128]. It recognises and binds specifically to the sequence GT-N_7-AC, which is repeated multiple times[129]. On the other hand, NsrR is a more global transcriptional factor regulating around 20 transcripts, as identified in several whole-genome analyses[130-133]. This protein is a transcriptional repressor of the Rrf2 family that senses NO through a [2Fe-2S] cluster[134]. When NO binds NsrR, this protein loses its ability to bind its target sequence, and stop repressing its target genes[103].

Proteins of the CRP superfamily also play important roles in sensing nitrosative stress and regulating gene expression in response[135]. Some examples of these proteins are Anr in *P. aeruginosa*[136], NnrR in *R. sphaeroides*[137], Nnr in *P. denitrificans*[138] and DnrD in *P. stutzeri*[139]. None of these proteins have the Cys residues necessary to bind the [4Fe-4S] centre that Fnr proteins use to sense oxygen. In many bacteria, regulation of nitrate reduction and response to nitrosative stress involve cascades of regulatory proteins or multiple CRP-related transcription factors, in contrast to what happens in *E. coli*, as we have seen above[103]. Moreover, Fnr proteins are also able to sense NO. In the absence of NO, Fnr proteins bind DNA and repress the expression of NO detoxification genes. In the presence of NO, the $[4Fe\text{-}4S]^{2+}$ cluster of Fnr becomes nitrosylated, forming a combination of monomeric and dimeric dinitrosyl-iron-cysteine complexes, becoming Fnr unable to bind DNA and thus releasing the repression of the NO detoxification genes[87,140]. Moreover, the inability of Fnr to bind DNA prevents the activation of all the anaerobiosis genes activated by it, also preventing the production of new NO from nitrate by the nitrate reductase until it is removed.

Bacteria have developed mechanisms to remove NO both in aerobic and anaerobic conditions. The flavohemoglobin Hmp is able to bind NO and oxidise NO to nitrate[103,141]. When Hmp is reduced, it donates an electron to NO, generating the nitroxyl anion NO^-, which then can react with O_2 and be oxidised to nitrate[103]. The purified protein was observed to be also able to reduce NO to N_2O, which would allow this protein to protect against nitrosative stress both in aerobic and anaerobic conditions[142]. However, in *E. coli* this enzyme is not kinetically competent to protect against nitrosative stress in anaerobic conditions as the turnover number for NO reduction is low and the K_m is too high[143].

An alternative way to reduce NO was the above mentioned flavorubredoxin NorV that is also able to reduce NO to N_2O[144-146]. However, although NorV is kinetically able to protect cells against NO, the NO-sensing enhancer NorR, that is essential for *norV* transcription, binds NO with low affinity, thus being the synthesis of NorV and its reductase NorW induced only when NO concentrations are high and nitrosative stress severe[147].

Another mechanism of NO detoxification, already mentioned in section 2.2.1., is the nitric oxide reductase Nor. Although all types of Nor are able to eliminate nitric oxide by its respiration to N_2O, the group named as qNor plays an important role in NO detoxification[67].

The periplasmic nitrite reductase NrfAB is able to reduce NO to ammonia using electrons from menaquinol[148]. However, this enzyme is only synthesised in anaerobic conditions, unlike other NO detoxification enzymes. Although V_{max} -maximum reaction speed- of this reaction is high enough to remove high concentrations of NO that enter the periplasm, K_m -substrate concentration required to reach half of V_{max}- is too high to prevent that NO enters the cytoplasm, thus being this enzyme effective just as an initial protection against exogenous NO[149].

Bacteria have also developed mechanisms to repair the damage caused by the presence of nitrosative stress. Proteins that contain surface-exposed [4Fe-4S] centres, such as the dehydratases aconitase and fumarase, are especially sensitive to inactivation by NO nitrosylation[150-152]. YtfE is a di-iron protein that

can transfer iron atoms to iron-deficient enzymes. However, it has been reported that this transference is very slow and that it needs stoichiometric quantities of YtfE, instead of catalytic quantities[153,154]. Furthermore, apart from this repair of iron centres (RIC) activity, YtfE can also act as a NO-trapping scavenger that facilitates its transformation to N_2O when NO flux is low, before nitrosative stress takes place[155].

3.3. Other detoxification systems

Hydrogen sulphide (H_2S) is a very toxic substance for aerobic organisms as it hinders oxygen transport and inhibits oxygen reduction by heme:copper oxygen reductases, not allowing energy production by oxidative phosphorylation. Moreover, it is a strong nucleophile that can react with disulphide bridges and bind to metal centres[156]. Heterotrophic bacteria are able to oxide sulphide to sulphite and thiosulphate using the enzimes sulphide:quinone oxidoreductase (SQR), persulphide dioxigenase (PDO) and a type of sulphurtransferase called rhodanese.

Bacterial SQRs are classified based on their structure into six types, from I to VI[157]. Type I SQRs are found in *Aquificaceae*, α- and β-Proteobacteria and cyanobacteria[158]. Type II are found in Bacteria (Firmicutes and Proteobacteria), including some pathogens as *Staphylococcus aureus* and *P. aeruginosa*, and in *Eukarya*, including yeast, worms, insects and mammals, but not plants[159]. Type III are the least characterised, and they belong to the green sulphur bacteria and to *Archaea*[157]. Type IV are mainly but not exclusively found in green sulphur bacteria, being one homologue in the purple sulphur γ-Proteobacterium *A. vinosum*[157]. Type V are archaeal proteins, majorly belonging to *Sulfulobales*[156]. Finally, type VI are a group of previously unclassified bacterial SQRs that include CT1087 from *Chlorobium tepidum* and Aq_788 from *Aquifex aeolicus*[157].

At least two different types of PDOs have been described in gram-negative bacteria: type I is an homolog of the ETHE1 from animals, plants and bacteria and type II consists of several reported proteins from Proteobacteria[160,161]. These two types use glutathione persulphide (GSSH) as substrate, but their substrate binding sites are different and they share low sequence similarity[162].

As for Gram-positive bacteria, the PDO of *S. aureus*, CstB, uses low-molecular weight persulphides (RSSH and RSS^-) as substrates, rather than glutathione persulphide[163].

Rhodaneses are present in animals, plants and microorganisms, and heterotrophic bacteria have usually a rhodanese domain fused to SQR or PDO[160,161,163,164].

In Gram-negative bacteria, SQR oxidises sulphide to polysulphide, which reacts spontaneously with GSH generating GSSH. Then PDO oxidises GSSH to sulphite, which spontaneously reacts with polysulphide producing thiosulphate. As for rhodaneses, they accelerate the reaction of polysulphide with GSH that produced GSSH, though this reaction can happen spontaneously[161].

3.4. SOS DNA damage system

When DNA damage occurs in the cell for different reasons such as oxidative[165,166] or nitrosative stress[167], and this damage has not been repaired by the conventional repair systems, resulting in structures that prevent normal DNA replication and double strand DNA breakage, bacteria have developed a series of emergency mechanisms to repair this damage, known as the SOS response[168].

In the absence of DNA damage, the different genes involved in the SOS response are repressed by the LexA protein dimer, that binds to the operator regions of these genes, the LexA boxes, and repress their transcription[169]. LexA protein consist of two domains separated by a flexible linker: the N-terminal DNA binding domain (NTD) and the C-terminal catalytic domain (CTD) with a serine-lysine catalytic dyad. In addition, CTD is responsible of homodimerisation of LexA[170,171]. The LexA box consensus was proposed to be 5' $CTGTN_8ACAG$ 3'[172], and for *E. coli* it can be expanded to 5' $TACTGT(AT)_4ACAGTA$ 3'[173,174]. The number of genes regulated by LexA varies depending on the species, being of about 40 genes in *E. coli*[175], 33 in *B. subtilis* (of which only 8 are homologous to those of *E. coli*)[176] and 15 in *P. aeruginosa*[177], to cite some examples. Moreover, LexA shows different affinity for these genes, depending

on the LexA box sequence and the number of LexA boxes present at the promoter region of each one.

When a double strand DNA break (DSB) or a double strand end (DSE) occurs, the RecBCD complex recognises it and, using its helicase and nuclease activity, generates a single strand DNA (ssDNA) substrate for the protein RecA. In the case of single strand nicks (gaps), the exonuclease RecJ transforms them in bigger gaps, so that they can be recognised by the RecFOR presynaptic complex, which recruits RecA to this ssDNA patch. Therewith, RecA binds the ssDNA forming a nucleofilament and catalyses the autoproteolysis of the repressor LexA near the middle of the protein[178], which leads to the de-repression of its regulon[179]. Furthermore, this proteolysis exposes residues that target LexA for degradation by proteases ClpXP and Lon[180]. LexA proteins which are bound to their target LexA box are insensitive to auto-proteolisis[181]. Therefore, the genes with LexA boxes that bind LexA with more affinity will be expressed later in time, if the SOS response continues, thus generating a sequential expression of different genes in this response.

The first mechanism activated in the SOS response is the homologous recombination, carried out by RecBCD, RecFOR and RecA, in which strands invasion with the sister chromosome results usually in a mutation-free repair of the damage[179]. The second mechanism, the nucleotide excision repair (NER) allows the repair of lesions in dsDNA, for example a mismatch[182]. In this mechanism, the UvrABD endonuclease is able to recognise the lesion and nick the DNA, then the UvrD helicase removes the DNA fragment carrying the lesion[183] and finally PolI DNA polymerase fills the gap, repairing the DNA[184]. The third mechanism activated is the translesion synthesis (TLS) in which the first proteins induced are the DNA polymerases PolII, coded by *polB* gene, and PolIV, coded by *dinB* gene. The cell division inhibitor SulA is also induced at this point, to give time to the bacterium to repair the damage. Finally, if the damage is too extensive and could not be repaired, the bacteria induced the expression of *umuC* and *umuD* genes, coding for the error-prone DNA polymerase PolV[179,185]. The RecA nucleofilament, similarly as with LexA, catalyses the proteolytic cleavage of UmuD, that becomes active[186]. This active UmuD forms a complex with UmuC, leading to the formation of the polymerase.

As this polymerase is error-prone, it leads to a high mutation rate in the cell, but allows replication and cell survival[175,187].

When the DNA damage is repaired, the disappearance of the induction signal of the SOS response allows LexA to accumulate once again, repressing the SOS genes[169].

4. Bacterial appendages

When bacteria encounter non-favourable or less favourable conditions, as anaerobiosis is for aerobic and most facultative anaerobic bacteria, they induce the expression of flagella and the whole motility and chemotaxis machinery in order to migrate to a more favourable environment[188]. For example, in enterobacteria and other bacteria, flagellar biosynthesis is increased in anaerobiosis and is regulated by ArcA in order to facilitate motility in search of nutrients[189-191]. However, in other bacteria, such as *P. aeruginosa* and *R. capsulatus*, a weak repression of some flagellar genes in anaerobic conditions has been observed[192,193]. Moreover, repression of flagellar genes has also been observed in *Sphingobium* cells at high concentrations of nickel, suggesting that this repression could be a response to stressing conditions[194].

Furthermore, type IV pili regulation has also been reported to be affected under stress conditions, *e.g*. in *E. coli*[195] under envelope stress or in *Thermus thermophilus* under temperature stress[196]. The expression of these pili is also affected in anaerobic conditions in many bacteria such as *Clostridium* sp. and *Pseudomonas extremaustralis*, as they play an important role in biofilm formation under anaerobiosis [197-199].

4.1. Structure and regulation of the bacterial flagella

4.1.1. Structure of the bacterial flagella

Bacterial flagellum is a complex structure consisting in a lot of different protein components. Figure 10 shows two different representations of the bacterial flagellum of *E. coli* which give complementary insights of its structure[200,201].

Introduction

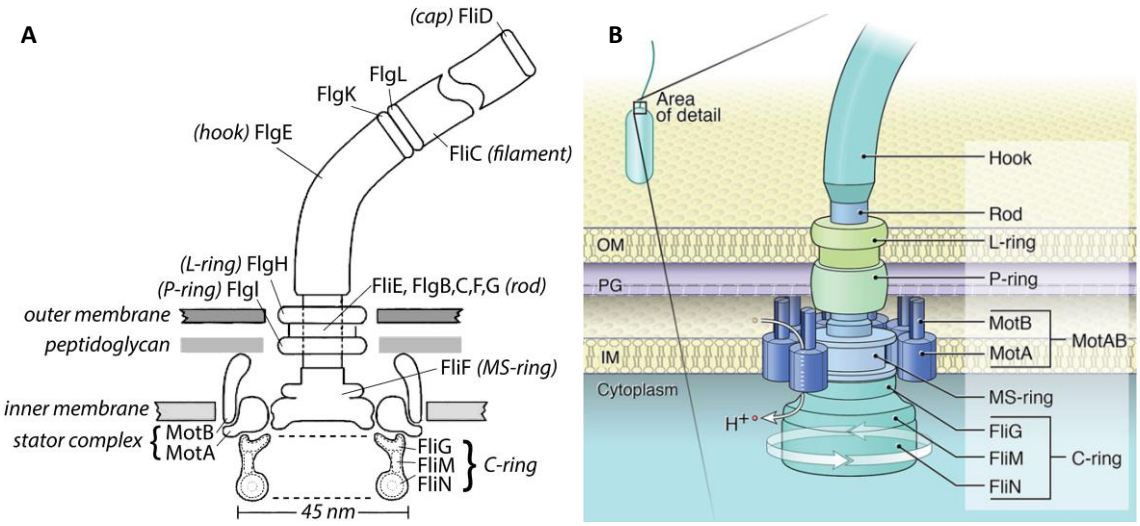


Figure 10. Flagellar structure. Two different representations of flagellar structure are shown. (A) 2D model of flagellar structure from a previous work[200].(B) 3D model of flagellar structure from a previous work[201].

These flagella are formed by external components, outside of the cell wall, and internal components, inside the cell wall. The external components include the filament (a polymer of the protein FliC, also known as flagellin[202,203]), the hook (FlgE)[204] and the hook-associated proteins (FlgK, FlgL and FliD) (Figure 10A). The two proteins FlgK and FlgL are adapter proteins that permit FlgE to flex and the filament to rotate rigidly[200,205]. FliD is a cap located in the distal end of the filament, under which the polymerisation takes place (Figure 10A)[206]. The filament is hollow along its whole length and comprises three axially connected tubes, and eleven protofilament strands can be observed in the cross section of the flagellin tube. The filament can be around 2000 layers of flagellin in length[207]. The hook consists of a short segment of right-handed helix formed by 12 ring-like layers of units of FlgE similarly to how the flagellins are arranged in the filament[207]. The hook, and therefore the filament, are connected to the internal components, that consist of several proteins and rings that are able to rotate, thus transferring this rotation to the filament (Figure 10)[208]. The filament's rotation is what propels the cell and this filament shows different polymorfic forms depending on the direction of the rotation and the torsion load. On the other hand, the hook needs to be a flexible joint as they project from the sides of the cells while the filaments are normally aligned to the long axis of the bacteria[200].

Among the internal components, there are 4 rings in a rod: the M ring, standing for membranous, the S ring for supramembranous, the P ring for peptidoglycan and the L ring for lipopolysaccharide[209]. One additional ring, the C ring (C standing for cytoplasmic), is located on the cytoplasmic side of the M ring[200]. These 5 rings form what is known as basal body. Some of the internal components are able to rotate: the rod, the MS ring and the C ring. The rod or drive shaft (composed of FliE, FlgB, FlgC, FlgF and FlgG) connects the hook and the MS ring, going through the L (FlgH) and the P (FlgI) rings, which are located in the outer membrane and the peptidoglycan layers respectively (Figure 10). The MS ring is the central part of the rotor and consists of multiple copies of the protein FliF[210]. Each stator complex, whose number can vary between 1 and 11, consists of 4 MotA and 2 MotB proteins (Figure 10B)[211]. They are linked to the peptidoglycan by the C terminus of MotB and interact electrostatically with FliG though a cytoplasmic domain of MotA[212,213]. The flow of protons through these stator complexes is what generates the torque, as it can be seen in Figure 10B)[214,215]. It is suggested that the ions passing these channels are bound by a conserved aspartic acid residue in the transmembrane region of MotB, leading to conformational changes in MotA[216]. These conformational changes are coupled to the rotation by the electrostatic interactions between MotA and FliG mentioned above[217,218]. The number of stator complexes depends on the viscosity of the surrounding environment and determines the rotational speed of the flagella, having being detected 11 different speed, one for stator complex[219,220].

The other components of the C ring, FliM and FliN, control de direction of rotation by interaction with phosphorylated CheY (CheY-P), a signalling protein of the chemotaxis network. In the absence of CheY-P and at room temperature, the direction of the rotation is counterclockwise[221]. As shown in Figure 10A, FliN tetramers resemble donuts in this cross-sectional view of the C-ring. The motor changes the number of FliM and FliN depending on the direction of rotation, being the lower number (34) found in clockwise-spinning motor and the higher one (45) in counterclockwise-spinning motors. This change on the number of these proteins takes place by exchange with the pools of free proteins in the cytoplasm[200,222].

As other elements not shown in Figure 10, we find FliH, an export component that interacts with FliN; FliL, that interacts with stator complex and the MS ring and enhances torque; and the flagellar export apparatus, which coordinates the export of axial components and is located in the centre of the cytoplasmic face of the MS ring[200].

The flagellum assembly is a sequential process that starts with the cytoplasmic parts and ends with the distal ones. This is regulated both by gene expression regulation and by posttranslational modifications of the flagellar proteins. The most important aspect is to control the order in which the components are transported through the inner channel of the flagellum.

The system that selects which component is transported is located in the cytoplasmic side of the flagellum and consist on the proteins FlhA and FlhB and an ATPase complex formed by the proteins FliH, FliI and FliJ (Figure 11)[223]. The hydrolysis of ATP is not necessary for transport but for selective delivery of transported substrates and likely for the partial unfolding of them[223,224]. The components of the flagellum are transported forming a complex with specific transport chaperones, interacting subsequently with the FliI subunit of the ATPase and being delivered to the FliM and FliN components of the C-ring. The FliJ component of the ATPase interacts with the chaperones that do not carry anything and prevents their binding to the components that were delivered to the base of the rod[224].

The first components that must be transported are those of the rod, followed by the hook, the adapter proteins FlgK and FlgL and, finally, the flagellin[207]. During the assembly of these tube-like parts, the end of each one is covered by a cap protein, FlgJ being the cap for the rod[225], FlgD for the hook[223] and the previously mentioned FliD for the filament. Only FliD cap remains in the final structure of the flagellum. Therefore, each cap must be transported before its tube component[207]. Although the mechanisms of switching from the assembly of the rod to the assembly of the hook, has not been deeply investigated, it is known that its length is limited by the regulation of FliK protein (Figure 11A, FliK is not shown). The P and L rings are exported though the Sec-dependent mechanism and Flk protein is the one in charge of regulation before the

assembly of this L-ring (Figure 11A)[226,227]. When the rod interacts with the outer L-ring the cap of the rod (FlgJ) is replaced by the cap of the hook (FlgD) (Figure 11B)[223]. FliK is then exported through the flagellar export system and its N-domain interacts with FlgD (Figure 11C)[228]. When the hook reaches the 55 nm, the C-domain of FliK interacts with FlhB and facilitates its proteolysis, triggering the switch of the mechanism of transport from early to late substrates (Figure 11D and E)[223,226,229]. This irreversibly halts the transport of the hook FlgE protein among other things and allows the transport of FlgM and other late substrates such as FliC to initiate (Figure 11F)[230,231]. The regulation of flagellar assembly by posttranslational changes provides an advantage as it allows the simultaneous assembly of several flagella in different stages of assembly.

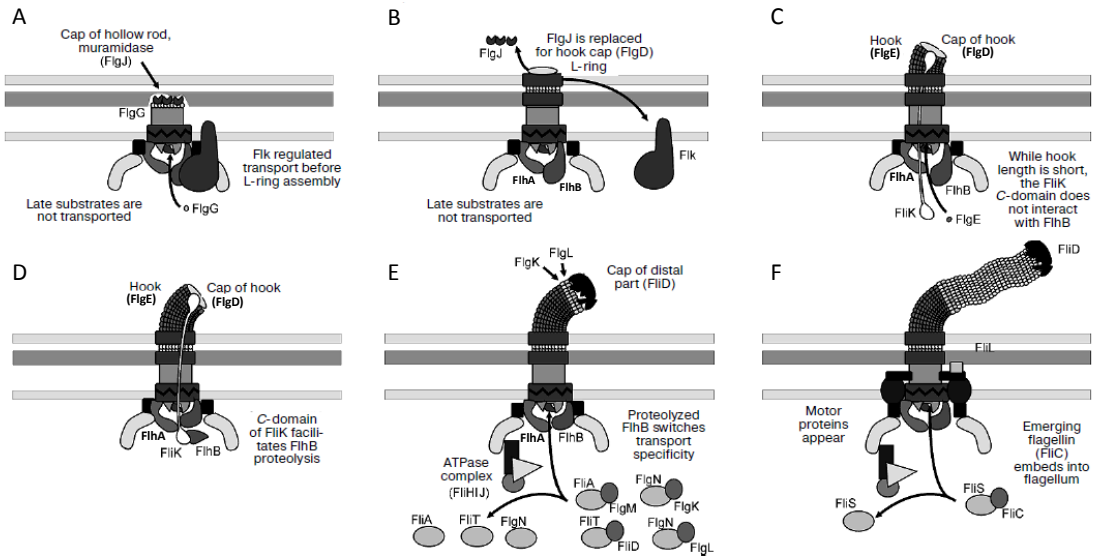


Figure 11. Flagellar assembly mechanism. The flagellar assembly mechanism steps are shown here. Figure adapted from a previous work[207].

4.1.2. Regulatory chains of flagellar biosynthesis

Flagellar regulation in bacteria is a complex process that involve several regulatory proteins and several promoters, which show a hierarchical relationship among them. In this hierarchy, the expression of the genes of each class depends on the expression of the genes of the previous class[232], thus forming an expression cascade of several tiers.

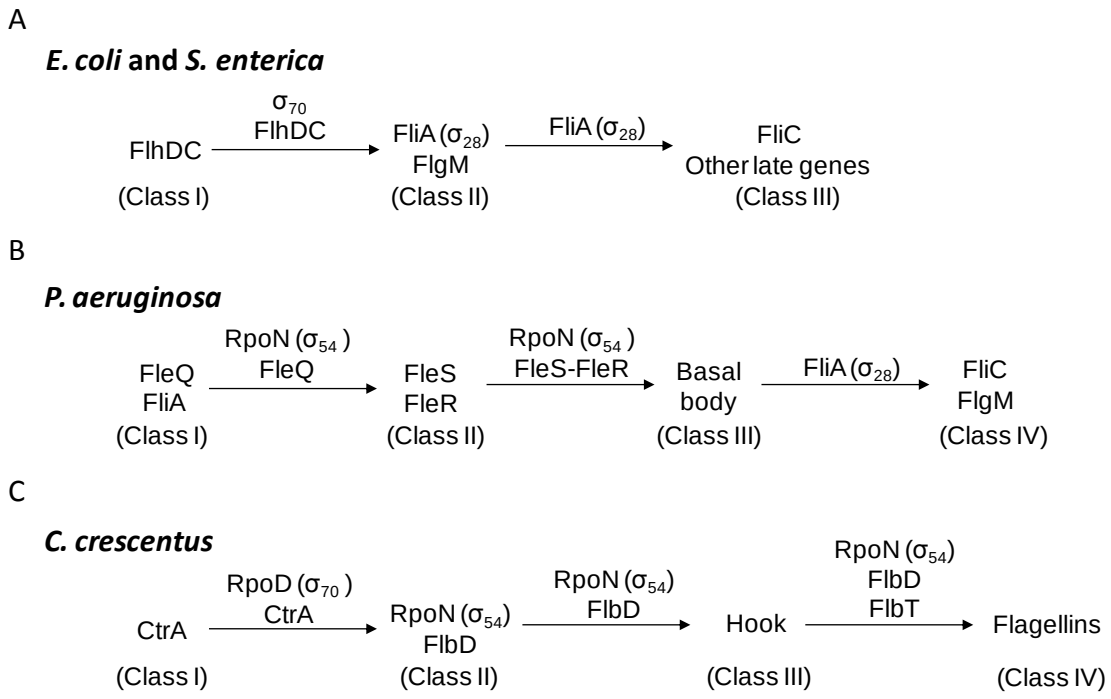


Figure 12. Flagellar regulation cascades of four bacterial species. Summary of three different flagellar regulation cascades, from *E. coli* and *S. enterica*[233] (A), *P. aeruginosa*[234] (B) and *C. crescentus*[235] (C).

In many bacteria as *E. coli* and *Salmonella enterica*, flagellar operons are divided in three temporally regulated transcriptional classes: early (class I), middle (class II) and late (class III) (Figure 12A). The class I promoter is a single promoter that transcribes the *flhDC* operon, coding for the two early proteins FlhD and FlhC, in response to many environmental cues. This class I promoter is a critical regulation point, in which the cell must decide to initiate or not the formation of new flagella. This decision is based in different global regulatory signals that include CRP regulators[236,237], temperature control[238,239], the heat shock proteins DnaK, DnaJ and GrpE[240], high concentrations of inorganic salts, carbohydrates or alcohols[241,242], DNA supercoiling[241-244], growth phase[245], surface-liquid transitions[246], phosphatidylethanolamine and phosphatidylglycerol synthesis[247,248], and cell cycle control[249-251]. These two proteins are transcriptional activators for σ^{70}-dependent class II promoters, where they form a heteromultimeric complex that facilitates the transcription of middle class genes, which include proteins necessary for the structure and assembly of the hook-basal body and the σ^{28} factor FliA and its anti-σ factor FlgM[252]. The class III promoters are specific for σ^{28} RNA polymerase and the gene products

necessary for late assembly stage, including the filament, with the exception of the hook-associated proteins, are transcribed only from these promoters[233]. The antisigma factor FlgM binds to the σ^{28} factor FliA, not allowing the expression of class III genes until the hook-basal body is complete. Once it is complete, FlgM is secreted from the cell and FliA is free to activate the expression of class III genes, so that the filament is not made until there is a hook-basal body to attach to. These transcriptional classes seem to correspond to the main steps in morphological development of the flagellum[253]. However, many genes are expressed from more than one promoter class, including *flgK*, *flgL*, *flgM*, *fliD*, *fliS* and *fliT*, that are transcribed from class II and class III promoters[254-256].

P. aeruginosa is a pathogenic γ-Proteobacteria producing a single polar flagella that is needed for virulence[257]. In contrast to *E. coli* and *S. enterica*, this bacterium shows four levels in its flagellar genes expression cascade (Figure 12B). *fleQ* and *fliA* are constitutively transcribed class I genes with regulatory functions. FleQ is the class I master regulator for flagellar synthesis, whose function is in turn regulated by global molecules like cyclic di-GMP, a signalling molecule that plays an important role in the transition between planktonic and biofilm lifestyles[258]. FleQ together with the σ^{54} factor RpoN are in charge of regulating the transcription of early class II flagellar genes, encoding components of the basal body and the filament cap, which is unexpected as the cap protein will not be needed until much later for filament bioynthesis[234]. Among these early transcribed genes also are *flhF*, *fleN*, *fleS* and *fleR*[234,259]. FleN is an antiactivator of FleQ that downregulates genes encoding early flagellar proteins in order to maintain the normal flagellum number of one per cell[260,261]. FleS and FleR form a two-component system necessary for the expression of the RpoN-dependent class III genes, coding for the rod, L ring, hook, hook cap, and hook-filament junction proteins. The constitutively transcribed Class I protein FliA is the sigma factor responsible for the expression of class IV late genes, which include flagellin FliC, the filament length control protein FleL, FlgM, FlgN (required for initiation of filament assembly) and some chemotaxis proteins. However, it has been reported that there is a basal transcription of FlgM, which is independent of RpoN, FleQ, FleR and FliA, depending on an unknown transcriptional or sigma factor. It is thought

that FlgM protein transcribed in a FliA independent way binds to FliA and inhibits its activity until the hook-basal body structure is complete. After its completion, FlgM is secreted from the cytoplasm, unbinding FliA and allowing it to activate class IV genes. This makes sure that Class IV genes are not transcribed too early, as their activator, FliA, is transcribed constitutively, as previously said[234].

In the α-Proteobacterium *Caulobacter crescentus*, the regulation cascade of flagellar genes is different, though it also consists of 4 hierarchical classes as in *P. aeruginosa* (Figure 12C). This bacterium divides asymmetrically producing a swarmer cell and a stalked cell, being the flagellar production coordinated with cell cycle so that all the daughter swarmer cells have flagella. Therefore, in *C. crescentus* the class I gene in the flagelar cascade is the cell cycle regulator CtrA[262]. Influence of the cell cycle regulator CtrA in flagellar production has also been reported in other α-Proteobacteria like *Magnetospirillum*[263], *R. sphaeroides*[264] and *Sphingomonas melonis*[265]. In *C. crecentus*, CtrA acts as a master regulator of flagellar gene expression inducing the transcription of early flagellar genes such as those encoding the MS ring, C ring, and export apparatus, FlbD and FliX[266]. CtrA also controls the expression of the gene *rpoD* encoding the σ^{70} factor[267]. FlbD and FliX are transcriptional regulators that control the expression of early and middle genes. FlbD, which activates promoters recognised by the RpoN σ_{54} factor, binds *ftr* (flagella transcriptional regulation) motifs in order to regulate gene expression, playing a dual role. On the one hand, once the early genes, which are not dependent on σ^{54}, are expressed, FlbD binds the *ftr* sequences in their promoters and inhibits their transcription. On the other hand, this protein binds to the *ftr* of the σ^{54}-dependent promoters for the middle genes and activate their transcription[268,269]. FliX regulates the activity of FlbD, inhibiting its binding to the *ftr* sites when the early flagellar structures are not finished and stimulating its binding when they are formed[270]. Finally, the expression of the 6 flagellin genes (class IV genes) of this bacteria is also σ^{54}-dependent, and regulated by FlbD, but additionally by FlbT, which binds to the 5' UTR of flagellin transcripts and represses their translation until the hook-basal body structure is assembled[271].

4.2. Bacterial fimbriae and their regulation

Besides flagella, some bacteria also show on their surface other proteinic appendages, generally known as pili. They can vary in length and are able to reach several microns or even exceed the diameter of the bacteria. These pili can have many functions in the cells: adherence and/or invasion of eukaryotic cells, biofilm formation through pili self-association, hollow conduit or scaffolding for secretion or uptake of proteins and nucleic acids, and even for extracellular electron transport in some electrogenic bacteria. Generally, pili involved in DNA transfer during conjugation between bacteria are known as sexual pili, whereas the rest of pili are known as fimbriae. Many different types of pilis have been described, especially in gram-negative bacteria, such as the chaperone-usher pilus system, type I, type II, type III, type IV, type V, bacterial amyloid fibers (curly and Fap fibers) and sortase mediated pili, among others[272]. Here we will focus on type IV pili, that are fimbriae.

Type IV pili are several micrometers long flexible appendages that are very extended as virulence factors among Gram-negative bacteria such as *P. aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningitidis* and *Myxococcus xanthus*. Some bacterial species are able to form bundle-forming pili in order to adhere to epithelial cells and for auto-aggregation, *e.g. Vibrio cholerae* and enteropathogenic *E. coli*[273]. Moreover, type IV pili have also been found in some Gram-positive bacteria, belonging to the *Clostridia* and *Ruminococcus* genera, in Cyanobacteria and in *Archaea*[274-276]. The ability of this type of pili to extend and retract by polymerisation and depolymerisation explains their role in adhesion to host cells and solid substrates, biofilm formation, DNA and phage uptake, cellular invasion and microcolony formation. Furthermore, this capability provide them with a flagella-independent movement, the twitching motility, consisting on repeated cycles of extension, adhesion and retraction, powered by ATPases[277].

The subunits of type IV pili present a methylated N-terminus, a hydrophobic 25-aminoacids N-terminal α-helix domain and a C-terminal disulphide bonded β-domain[278]. Type IV pilins are divided in two categories, according to their length and sequence similarities. Type IVa pilins are shorter than type IVb, being the

first around 150 aminoacids long while the second are around 190 aminoacids long, and show a shorter signal peptide, 5-6 aminoacids in contrast to the 15-30 aminoacids of type IVb[279,280]. Furthermore, the N-methylated residue of type IVa is always phenylalanine while in type IVb it can be either methionine, leucine or valine. Also, different protein topologies of the β-domain of types IVa and IVb have been observed in pilin structures avaliable[281]. Type IVa pili can be found in many Gram-negative bacteria, some of them being pathogens, like *Neisseria* ssp. or *P. aeruginosa*, but type IVb pili have only been identified in human enteric bacteria like *V. cholerae*, *S. enterica* serovar typhimurium, and enteropathogenic and enterotoxic *E. coli*. Therefore, the host range of cells having type IVa pili is broader than that of the ones having type IVb and can include several mammals, plants and even other bacteria[278]. Type IVa pili require for their assembly a complex machinery consisting of many components that are coded in different parts of the bacterial genome. On the contrary, type IVb pili assembly system consists of less proteins that are generally coded in gene clusters, sometimes in plasmids[282]. Besides the differences between them, both type IVa and IVb pilins have a very similar architecture. They are small proteins of around 15-20 kDa with an extended hydrophobic N-terminal α-helical domain (α1) and a globular C-terminal domain[272].

As for the regulation of flagellar transcription, in many bacteria, the pilin subunits are expressed from a σ_{54} promoter. The complex formed by RpoN and RNA polymerase bind the promoter but remain in a closed configuration until the enhancer binding protein PilR, the response regulator of the two-component system PilR-PilS, contacts and activates it[281]. The expression of pilin gene is regulated in response of several environmental cues, depending on the lifestyle of the organism. These cues include surface contact, local increase in viscosity or mechanical strain on membranes[283], pH[283], UV radiation[284,285], temperature, culture conditions or host cell contact[286-288], among other.

OBJECTIVES

Objectives

1. To determine whether *Sphingopyxis granuli* strain TFA is able to grow in anaerobic conditions by respiration and/or fermentation, and the possible final electron acceptor that it is able to use.

2. To analyse expression pattern of genes in anaerobic conditions or contrast to aerobic conditions, and to identify genes that are differentially regulated.

3. Identification of the regulatory proteins involved in the adaptation from aerobic to anaerobic conditions.

4. To identify target genes directly controlled by these anaerobic regulatory proteins in order to establish their direct regulon.

MATERIALS AND METHODS

1. Strains, plasmids, primers and growth conditions

1.1. Strains and plasmids

Strains and plasmids used in this thesis are listed and described in the following tables 1 and 2.

Strain	Description	Reference
***Sphingopyxis granuli* TFA**		
TFA	Wild type strain. Sm^R.	Hernáez *et al*., 1999[2]
MPO250	Deletion mutant Δ*fnrN.* Deletion of 688 bp out of 696 bp. Sm^R.	This work
MPO251	Deletion mutant Δ*fixK.* Deletion of 677 bp out of 723 bp. Sm^R.	This work
MPO252	Double deletion mutant Δ*fnrN*Δ*fixK.* Deletion of 688 bp out of 696 bp in *fnrN* and of 677 bp out of 723 bp in *fixK*. Sm^R.	This work
MPO253	Deletion mutant Δ*narG.* Deletion of 3624 bp out of 3744 bp. Sm^R.	González-Flores *et al*., 2019[289]
MPO254	Deletion mutant Δ*fleQ.* Deletion of 1125 bp out of 1146 bp. Sm^R.	This work
MPO255	Spontaneous mutant that swims faster. Sm^R.	This work
MPO256	Deletion mutant Δ*ctrA.* Deletion of 681 bp out of 708 bp. Sm^R.	This work
MPO851	Deletion mutant Δ*fliA.* Deletion of 675 bp out of 744 bp. Sm^R.	Constructed in our lab by Manuel Cabrera
MPO804	Insertion of Ap^R gene in *fixJ1* gene and substitution of *fixJ2* gene by a Km^R gene. Sm^R, Ap^R, Km^R.	Gómez Alvarez, 2014[290]
MPO808	Substitution of *fixL2* and *fixJ2* genes by a Km^R gene. SmR, KmR.	Gómez Alvarez, 2014[290]
MPO811	Substitution of *fixL1* and *fixJ1* genes by a Ap^R gene. Sm^R, Ap^R.	Gómez Alvarez, 2014[290]
MPO812	Double mutant with the substitutions of mutants MPO808 and MPO811, resulting in a strain lacking *fixL1*, *fixJ1*, *fixL2* and *fixJ2* genes. Sm^R, Ap^R, Km^R.	Gómez Alvarez, 2014[290]
Escherichia coli		
DH5α	*supE44*, Δ*lacU169* (*φ80 lacZΔM15*), *hsdR17* (*rk-mk+*), *recA1*, *endA1*, *thi1*, *gyrA*, *relA*	Hanahan, 1983[291]
DH5α λpir	λpir lysogen of DH5α	Lab collection
M182	Δ(*lac IPOZYA*)- *X74 galU galK strAr*	Casadaban *et al*., 1980[292]
JRG6348	Derivative of JRG1728 lac, *fnr* carrying a chromosomal FNR-dependent promoter-lacZ fusion. Cm^R.	Dr. David Lee (University of Birmingham, UK)

Table 1. Bacterial strains used in this thesis.

Name	Description	Reference
pIZ1016	Derivative of pBBR1MCS-5 plasmid with *ptac* promoter and *lacIq*. Gm^R.	Martínez-Pérez *et al*., 2004[293]
pGS2350	pBAD-HisB (Invitrogen) derivative for expression of *E. coli fnr*. Ap^R.	Ibrahim *et al*., 2015[294]
pSW-I	*oriRK2*, *xylS*, *Pm→I-sceI* (transcriptional fusion of *I-sceI* to *Pm*). Ap^R.	Wong and Mekalanos, 2000[295]
pEMG	KmR, *oriR6K*, *lacZα* with two flanking I-SceI sites. Km^R.	Martínez-García *et al*., 2011[296]
pMPO704	TFA *fnrN* gene cloned in pIZ1016 under ptac promoter. Gm^R.	This work
pMPO705	TFA *fixK* gene cloned in pIZ1016 under ptac promoter. Gm^R.	This work
pMPO1412	Plasmid derived from pEMG, with a Sm^S gene inserted. Km^R, Sm^S.	González-Flores *et al*., 2019[289]
pSEVA224	*oriRK2*, *lacIq-PtrC*. Km^R.	Silva-Rocha *et al*., 2013[297]
pMPO706	TFA *fleQ* gene cloned in pSEVA224 under *ptrC* promoter. Gm^R.	This work
pMPO707	pEMG plasmid with *fnrN* flanking regions cloned in the MCS. Km^R, Sm^S.	This work
pMPO708	pEMG plasmid with *fixK* flanking regions cloned in the MCS. Km^R, Sm^S.	This work
pMPO709	pMPO1412 plasmid with *narG* flanking regions cloned in the MCS. Km^R, Sm^S.	González-Flores *et al*., 2019[289]
pMPO710	pMPO1412 plasmid with *fleQ* flanking regions cloned in the MCS. Km^R, Sm^S.	This work
pMPO711	pMPO1412 plasmid with *ctrA* flanking regions cloned in the MCS. Km^R, Sm^S.	This work
pMPO738	pMPO1412 plasmid with *fliA* flanking regions cloned in the MCS. Km^R, Sm^S.	Constructed in our lab by Manuel Cabrera
pUC18	Clonation vector. Ap^R.	Norrander *et al*., 1983[298]
pUC19	Clonation vector. Ap^R.	Norrander *et al*., 1983[298]

Table 2. Plasmids used in this thesis.

1.2. Primers

Primers used in this thesis are listed and described in Table 3.

Name	Sequence 5'--> 3'	Use
Primers for clonations		
fnrLF1F	CCCATCACGAATTCGCCATGCC	Forward primer for the amplification of the upstream region of *fnrN* gene in TFA
fnrLF1R	CATCGCAGGGATCCATGATTTAAAG	Reverse primer for the amplification of the upstream region of *fnrN* gene in TFA

fnrLF2F	CACTCGCCGGATCCTCAGAAGCG	Forward primer for the amplification of the downstream region of *fnrN* gene in TFA
fnrLF2R	GACGATTCTCTAGAAGACGGTCG	Reverse primer for the amplification of the downstream region of *fnrN* gene in TFA
fixKF1F	GAAGCGCGAATTCTATGCCCG	Forward primer for the amplification of the upstream region of *fixK* gene in TFA
fixKF1R	CTTCGTCAGGATCCGCTTTCCCG	Reverse primer for the amplification of the upstream region of *fixK* gene in TFA
fixKF2F	CGAGATTCTGGATCCACGCGGG	Forward primer for the amplification of the downstream region of *fixK* gene in TFA
fixKF2R	GGCACCATCTAGAAGGCCGCC	Reverse primer for the amplification of the downstream region of *fixK* gene in TFA
narGF1F	CAAAGCTTGGATCCGTCTTGCG	Forward primer for the amplification of the upstream region of *narG* gene in TFA
narGF1R	TCGGGGATCCTTTGCTTCGTCTTG	Reverse primer for the amplification of the upstream region of *narG* gene in TFA
narGF2F	AATATGGGATCCTCCGCAAATTG	Forward primer for the amplification of the downstream region of *narG* gene in TFA
narGF2R	GCAGGTTGTCTAGATATTTGAGC	Reverse primer for the amplification of the downstream region of *narG* gene in TFA
nifA6F1F	GGTGAATTCGCTCATCATCGCC	Forward primer for the amplification of the upstream region of *fleQ* gene in TFA
nifA6F1R	GATTATGGATCCCGACATAACCC	Reverse primer for the amplification of the upstream region of *fleQ* gene in TFA
nifA6F2F	AAATATGGGATCCACCAACAGG	Forward primer for the amplification of the downstream region of *fleQ* gene in TFA
nifA6F2R	GTCGAGAAGCTTTTCAACCCG	Reverse primer for the amplification of the downstream region of *fleQ* gene in TFA

ctrA F1 Fw	ATATGAATTCCGTGTTGCAGCCAAGATAGC	Forward primer for the amplification of the upstream region of *ctrA* gene in TFA
ctrA F1 Rv	ATATGGTACCCAGTACGCGCATTGGGCAC	Reverse primer for the amplification of the upstream region of *ctrA* gene in TFA
ctrA F2 Fw	ATATGGTACCGTCGCCTGACGCCGCATC	Forward primer for the amplification of the downstream region of *ctrA* gene in TFA
ctrA F2 Rv	ATATTCTAGAAAGGCTGCGCTCGGGTGC	Reverse primer for the amplification of the downstream region of ctr*A* gene in TFA
fliA1	TAACTCGAGATCGGCTATGCGC	Forward primer for the amplification of the upstream region of *fliA* gene in TFA
fliA2	ATAGGATCCTCATGCTGATGCTCCT	Reverse primer for the amplification of the upstream region of *fliA* gene in TFA
fliA3	TATGGATCCAGATCAAGCGCGAC	Forward primer for the amplification of the downstream region of *fliA* gene in TFA
fliA4	TATTCTAGACGTTCTGCTCATCACGG	Reverse primer for the amplification of the downstream region of *fliA* gene in TFA
fnrLHindIIIF	GATGGAAGCTTTAAATCATGAATTC	Forward primer for the amplification of *fnrN* gene
fnrLXbaIR	CTGCGCTCTAGATCAGCCGGCG	Reverse primer for the amplification of *fnrN* gene
fixKPstIF-2	ATCGGACTGCAGTTTGCGATTGC	Forward primer for the amplification of *fixK* gene
FixKXbaIR-2	GCGGAGCGTCTAGAGGGGCCTG	Reverse primer for the amplification of *fixK* gene
nifA6 F2	CATGAATTCAATCTGGCAGCATATCGGACG	Forward primer for the amplification of *fleQ* gene
nifA6 Rv3	CATGCATGCAGCCCCTTGTTTCAGCGGTG	Reverse primer for the amplification of *fleQ* gene
Primers for RT-qPCR		
narG qPCR F2	GCTGACCTGGTTCGTCTAC	Forward primer for *narG* gene RT-qPCR
narG qPCR R2	AGCTGGCTCATGCGGAAAC	Reverse primer for *narG* gene RT-qPCR
cyoC qPCR F	GAGCCGGTGTCCTTCTATC	Forward primer for *cyoC* gene RT-qPCR
cyoC qPCR R	CCGCCGAGCACCGCATAG	Reverse primer for *cyoC* gene RT-qPCR
cydA qPCR F	TCCAGTTCGGCACCAACTG	Forward primer for *cydA* gene RT-qPCR

cydA qPCR R	GCTTGTCCCATCCGAAGAAC	Reverse primer for *cydA* gene RT-qPCR
ccoH qPCR F	GTTTCTTCGGCACGATCATC	Forward primer for *ccoH* gene RT-qPCR
ccoH qPCR R	CATCGTCGAGCCACTTGTTG	Reverse primer for *ccoH* gene RT-qPCR
aox qPCR F	CGCGGTCGTGCTCGAAAC	Forward primer for *aox* gene RT-qPCR
aox qPCR R	GTCTTGATCCAGCCCTTGTC	Reverse primer for *aox* gene RT-qPCR
yhbU qPCR F	GGCAGCCCGGAGCTCATC	Forward primer for *yhbU* gene RT-qPCR
yhbU qPCR R	TGAAGTTGAGGCCGGAGAAG	Reverse primer for *yhbU* gene RT-qPCR
ahpC2 qPCR F	TCCATGCCGCGGATCAAC	Forward primer for *ahpC2* gene RT-qPCR
ahpC2 qPCR R	GTGCAGACCGGCGTGAAG	Reverse primer for *ahpC2* gene RT-qPCR
lsfA qPCR F	CAGGCCGCACCCGATTTTG	Forward primer for *lsfA* gene RT-qPCR
lsfa qPCR R	GTGCAGATCGGCGTATAATC	Reverse primer for *lsfA* gene RT-qPCR
ectA qPCR F	GATGCCGGGTCTGCAACTG	Forward primer for *ectA* gene RT-qPCR
ectA qPCR R	ATGCACTGATCGGCGAAATG	Reverse primer for *ectA* gene RT-qPCR
ytfE qPCR F	GCAAGCAATCCCGTCGAAAC	Forward primer for *ytfE* gene RT-qPCR
ytfE qPCR R	GACGCGCTGTGCCATCTG	Reverse primer for *ytfE* gene RT-qPCR
norB qPCR F	GGGGATTTCCGGGGTAGAG	Forward primer for *norB* gene RT-qPCR
norB qPCR R	ATCGAGCCGAGCTGCATTC	Reverse primer for *norB* gene RT-qPCR
ccrM qPCR F	GCATCCTGCAGGGCGATTG	Forward primer for *ccrM* gene RT-qPCR
ccrM qPCR R	GAGGTCGCCGCCGAGTTG	Reverse primer for *ccrM* gene RT-qPCR
ctrA qPCR F	GACGACCGAGGGCTTCAAC	Forward primer for *ctrA* gene RT-qPCR
ctrA qPCR R	CAATTTCTTGAGCACGTCATAG	Reverse primer for *ctrA* gene RT-qPCR
nrdZ qPCR F	CTGCGAGCGGGAGTTTCTG	Forward primer for *nrdZ* gene RT-qPCR
nrdZ qPCR R	GTCGAGATCGTCGGGAATC	Reverse primer for *nrdZ* gene RT-qPCR
recA qPCR F	ACAATTGTCACTCGTCGAATC	Forward primer for *recA* gene RT-qPCR
recA qPCR R	CTTCGAGCCGAGCTTCATC	Reverse primer for *recA* gene RT-qPCR
imuA qPCR F2	GACGCGCTTCGATCTTTATG	Forward primer for *imuA* gene RT-qPCR
imuA qPCR R2	AGGTCGCGGTCGGATCTG	Reverse primer for *imuA* gene RT-qPCR
fliC qPCR F	ACTGTCATCAACACCAATGTG	Forward primer for *fliC* gene RT-qPCR
fliC qPCR R	GTCCTTCGCGCTGTTGATG	Reverse primer for *fliC* gene RT-qPCR

cpaA qPCR F	GATCTCGAACGGGCTGAAC	Forward primer for *cpaA* gene RT-qPCR
cpaA qPCR R	CGGCGAACAGCAGGAATAC	Reverse primer for *cpaA* gene RT-qPCR
fixK qPCR F	CGAGACGCTGGCCGATTTC	Forward primer for *fixK* gene RT-qPCR
fixK qPCR R	TCCGACAGGATCGCGTAAAG	Reverse primer for *fixK* gene RT-qPCR
fnrN qPCR F	GACTCCGTCGAGGTTGAG	Forward primer for *fnrN* gene RT-qPCR
fnrN qPCR R	GTACCGGTGGACAATTTGAG	Reverse primer for *fnrN* gene RT-qPCR
nnsR2 qPCR F	CGCCTACCGCCTGTTCTTC	Forward primer for *nnsR2* gene RT-qPCR
nnsR2 qPCR R	ACAGGGTGACGACGATGAC	Reverse primer for *nnsR2* gene RT-qPCR
0596 qPCR F	GCAGATGGGCAAGCCTTTG	Forward primer for SGRAN_0596 gene RT-qPCR
0596 qPCR R	CGGCAGCCTTGATCTCATAG	Reverse primer for SGRAN_0596 gene RT-qPCR
0597 qPCR F	CTGCCATGTTCGGATTCAAG	Forward primer for SGRAN_0597 gene RT-qPCR
0597 qPCR R	CGCCTTGCCGCCCTTCAG	Reverse primer for SGRAN_0597 gene RT-qPCR
1383 qPCR F	GATTCGCCCCATGTGCATTC	Forward primer for SGRAN_1383 gene RT-qPCR
1383 qPCR R	GCGGCAGCAACAGGCAATG	Reverse primer for SGRAN_1383 gene RT-qPCR
2501 qPCR F	CGACGAGGAAGCGGTACAG	Forward primer for SGRAN_1431, SGRAN_2501 and SGRAN_3211 genes RT-qPCR
2501 qPCR R	CCGCTTCCACGACGCAATC	Reverse primer for SGRAN_1431, SGRAN_2501 and SGRAN_3211 genes RT-qPCR
Other primers		
aguBF	GCAATTTGTCCAAGGCGTTCGG	Primer forward that anneals upstream of *fnrN* used for several checks
M13 Rv	TCACACAGGAAACAGCTATGAC	Primer reverse that anneals in pEMG derivatives used to check integration of these derivatives in TFA
pSW-F	GGACGCTTCGCTGAAAACTA	Diagnose loss pSW plasmid
pSW-R	AACGTCGTGACTGGGAAAAC	Diagnose loss pSW plasmid

KmFw-pk18	GATTGAACAAGATGGATTGC	Primer forward that amplifyes the Km gene of pEMG derivatives used to check integration of these derivatives in TFA
KmRv-pk18	CGTCAAGAAGGCGATAGAAGG	Primer reverse that amplifyes the Km gene of pEMG derivatives used to check integration of these derivatives in TFA
rpsl1 Fw	GTTCGCTAGGGCCCATGG	Primer forward to amplify the streptomycin sensitivity gene of pMPO1412 plasmid
rpsl1 Rv	AATCCTGCTCTGCGAGGC	Primer reverse to amplify the streptomycin sensitivity gene of pMPO1412 plasmid
narG qPCR R	CAGGAGCCGGTGCAGTTC	Reverse primer used for comprobation of Δ*narG* mutant
nifA6 R2	CATGCATGCTCGACACGAACGGATAAGGG	Reverse primer used for comprobation of Δ*fleQ* mutant
phaC1	GACGAACAGCTTGAGGTC	Primer forward amplifying a fragment of *phaC* gene of TFA used to see absence of TFA genomic DNA in RNA samples
phaC4	AAGGGGCTGAAGCACATGC	Primer reverse amplifying a fragment of *phaC* gene of TFA used to see absence of TFA genomic DNA in RNA samples
FfixKCompF	CATCGCTATTGGCCGGCCCG	Primer forward that anneals in the upstream region of *fixK* used to check the integration of pMPO708
Comp mut ctrA Fw	TATCTCGCGAGCCACGGC	Primer forward that anneals in the upstream region of *ctrA* used to check MPO256 mutant
Seq F1 ctrA Rv	CGTAAATCAGCCCTTGGGAG	Primer forward that anneals in the downstream region of *ctrA* used to check MPO256 mutant

Table 3. Primers used in this thesis.

1.3. Media and growth conditions

Aerobic and anaerobic growth curves

Escherichia coli strains were cultured in Luria-Bertani medium (LB)[299] both in liquid cultures and plates, adding the corresponding antibiotics (see table 4) and

other compounds when necessary. They were growth at 37 ºC and with an agitation of 180 rpm in the case of liquid cultures.

LB: Bacto tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L. For solid LB 15 g/L agar were added. Autoclave.

S. granuli TFA strains were cultured in rich medium MML[2] or mineral medium (MM)[1] supplemented with β-hydroxybutirate (β-HB) 40 mM as carbon source, adding the corresponding antibiotics (see table 4). For anaerobic growth curves, several different final electron acceptors were tested (Table 5). Cultures were grown at 30 ºC and with an agitation of 180 rpm in the case of liquid aerobic cultures. Cell concentration in liquid cultures was determined by measurement of the Absorbance at 600 nm (Optical Density at 600 nm, OD_{600}). Some aerobic growth curves were performed in a Synergy HTX Multi-Mode Reader, where cultures were incubated in 96-well plates and OD_{600} was measured automatically.

For anaerobic growth in plates for electron microscopy, and western blots, plates were introduced in anaerobic jars and the air inside the jar was substituted by an atmosphere with 8% CO_2, 0.2% O_2 and the remaining volume completed with N_2. Growth was carried out with the presence of an active carbon catalyser, which would remove the remaining low oxygen concentration.

	Concentration in media (mg/L)	
Antibiotic	***E. coli***	***S. granuli***
Streptomycin	50	50
Kanamycin	25	20
Ampicillin	100	5
Chloramphenicol	15	-
Gentamycin	10	10

Table 4. Antibiotic concentrations added in the culture media for regular growth of *S. granuli* and *E. coli*.

Final electron acceptor	Concentration (mM)
Nitrate	20 and 40
Fumarate	10 and 40
Nitrite	3, 5, 10 and 50
DMSO	10

Table 5. Final electron acceptors and concentrations tested in TFA in anaerobic conditions.

MML: Bacto tryptone 2 g/L, yeast extract 1 g/L. . For solid MML 15 g/L agar were added. Autoclave. Add solution 1 at 2% (v/v) and solution 2+3 at 2% (v/v).

TFA MM: Milli-Q water. For solid MM 15 g/L agar were added. Autoclave. Add solution 1 at 2% (v/v) and solution 2+3 at 2% (v/v) and β-HB 40 mM.

Solution 1: $Na_2HPO_4 \cdot 12H_2O$ 3 % (w/v), K_2HPO_4 5 % (w/v). Autoclave.

Solution 2: $Ca(NO_3)_2 \cdot 4H_2O$ 0.25 % (w/v), $(NH_4)_2SO_4$ 5 % (w/v), $MgSO_4 \cdot 7H_2O$ 1 % (w/v), Ammonium iron(III) citrate 0.05 % (w/v). Autoclave.

Solution 3: $ZnSO_4 \cdot 7H_2O$ 0.1 % (w/v), $MnCl_2 \cdot 4H_2O$ 0.03 % (w/v), H_3BO_3 0.3 % (w/v), $CoCl_2 \cdot 6H_2O$ 0.2 % (w/v), $CuCl_2 \cdot 6H_2O$ 0.01 % (w/v), $MaMoO_4 \cdot 2H_2O$ 0.03 % (w/v). Filtrate.

Solution 2+3: Mix 190 mL of solution 2 with 10 mL of solution 3.

Fuctional complementation curves

For functional complementation curves of TFA Δ*fnr* mutants with TFA *fnr* genes both rich MML medium and TFA mineral medium with β-HB 40 mM as carbon source and nitrate 20 mM as final electron acceptor was used.

For functional complementation curves of *E. coli* strains with TFA *fnrN* and *fixK* genes a mineral medium (MM) [292] with casamino acids 0.5 g/L, the non-fermentable carbon source glycerol 40 mM and sodium nitrate 20 mM was used.

E. coli MM: NaCl-P 1x, NH_4Cl 1 g/L, microelements 1x, vitamins 1x.

Materials and methods

NaCl-P 10x: Na_2HPO_4 · 12 H_2O 140.73 g/L, KH_2PO_4 28 g/L, NaCl 5 g/L. Autoclave.

Microelements 100x: $MgSO_4$ · 7 H_2O 50 g/L, $FeSO_4$ · 7 H_2O 5 g/L, $MnSO_4$ · H_2O 2.5 g/L, $ZnCl_2$ 3.2 g/L, $CaCl_2$ · 2 H_2O 0.33 g/L, $CuSO_4$ · 5 H_2O 0.18 g/L, $CoCl_2$ · 6 H_2O 0.15 g/L, H_3BO_3 3.25 g/L, EDTA 5 g/L, 2.7 % HCl (w/v). Filtrate.

Vitamins 100x: Thiamin-HCl 10 mg/L, Biotin 4 mg/L, folic acid 4 mg/L, nicotinamide 20 mg/L, pyridoxine-HCl 20 mg/L. Filtrate, protect from light and store at 4 ºC.

Curves for high-throughput RNA sequencing

For sample preparation high-throughput RNA sequencing by dRNA-Seq for all growth conditions, TFA was previously grown aerobically at 30°C in mineral medium containing 40 mM β-HB as the only carbon and energy source to the exponential phase (OD_{600}=0.8). Then, cells were diluted into a fresh medium to an initial optical density of about 0.1. For TFA strain growing in aerobic conditions, cultures were grown at 30 ºC with 180 rpm of agitation and pellets were collected and frozen at OD_{600} of 0.7-0.8. For TFA strain growing in anaerobic conditions, cultures were transferred into standing stoppered bottles filled to the top and with sodium nitrate 20 mM as a final electron acceptor and grown at 30 ºC without agitation and pellets were collected and frozen again at OD_{600} of 0.7-0.8. For the cultures in presence of DETA-NO, cultures were grown aerobically at 30 ºC and 180 rpm until OD_{600} of 0.7-0.8. Then 4 mM of the NO releaser was added to the cultures, they were incubated in the same conditions for one additional hour, and then pellets were collected and frozen. For tetralin-grown cells, tetralin was supplied as unique carbon source in the gas phase[300] and cultures were grown aerobically at 30 ºC and 180 rpm until OD_{600} of 0.7-0.8, when pellets were collected and frozen. In the case of MPO252 (Δ*fnrN*Δ*fixK*) and MPO253 (Δ*narG*) mutants in anaerobic conditions, cells were grown first aerobically at 30 ºC and 180 rpm agitation until they reached an OD_{600} of 0.6-0.7. Then sodium nitrate was added to the cultures, they were transferred into standing stoppered bottles filled to the top and incubated at 30 ºC without agitation for 6 hours, when the pellets were collected and frozen.

Curves for RT-qPCR

To validate the results obtained by dRNA-seq and to study the expression levels of selected genes along the growth curves in these and other conditions, RT-qPCR in these curves were performed.

For TFA strain growing in aerobic conditions and anaerobic conditions with nitrate 20 mM, cells were grown aerobically in mineral medium with 40 mM β-HB until exponential phase and then diluted to an initial optical density of about 0.1 in the same media. After one hour of aerobic incubation, the initial time 0 was taken, the culture was split, sodium nitrate was added to the one half of the culture and then incubated under anaerobiosis during the rest of the assay. The other half was incubated under aerobic conditions. Samples were taken at different times and pellets were collected and frozen.

For TFA strain in anaerobic conditions without nitrate 20 mM with or without DETA-NO, and for MPO252 (Δ*fnrN*Δ*fixK*) and MPO253 (Δ*narG*) mutants in anaerobic conditions with nitrate, cells were grown in mineral medium with 40 mM β-HB in aerobic conditions the exponential phase and then diluted to an initial optical density of about 0.1 in the same media. Cells were then grown until an optical density of about 0.7, time 0 was taken and cultures were incubated under anaerobic conditions either in the absence of nitrate with or without 4mM DETA-NO for the wild type, or with nitrate for MPO252 and MPO253 mutants. Samples were taken at different times and pellets were collected and frozen.

Three independent biological replicates of each growth condition were frozen and treated for RNA purification as explained below.

1.4. Biological material preservation

All bacterial strains of TFA and *E. coli* were stored frozen at -80 ºC in rich medium, LB for *E. coli* and MML for TFA, with glycerol 15% (v/v). Plasmids were conserved the same way inside *E.* coli DH5α, or DH5α λpir strain in the case of pEMG and its derivatives.

Materials and methods

2. DNA transfer to bacterial strains

2.1. *Escherichia coli* transformation by heat shock

Competent cells preparation

For transformation of DH5α with plasmids and ligations, competent cells were prepared using a previously described method[301] with which a high transformation efficiency of up to 10^9 transformant cells per µg plasmid is achieved. In this method, a saturated culture of DH5α was diluted 100-fold in 200 mL of SOB medium and incubated at 22 ºC with agitation until OD_{600} 0.5. The culture was immediately introduced in ice where it was kept for 10 minutes. It was then centrifuged at 2500 g and 4 ºC for 10 minutes, the supernatant was discarded and the cell pellet resuspended in 20 mL of cold TB, to which 60 mL more of cold TB was added. After a 10 minutes incubation, cell were centrifuged once again under the same conditions, being resuspended in 20 mL of cold TB this time. Finally, 1.5 ml DMSO was added and cells incubated in ice for 10 minutes. Aliquots of 100 µL were prepared, frozen using liquid nitrogen and stored at -80 ºC until use.

SOB medium: tryptone 2% (w/v), yeast extract 0.5% (w/v), NaCl 8.5 mM, KCl 1.25 mM. Adjust pH to 2.0 with NaOH and autoclave. Subsequently add $MgCl_2$ 10 mM and glucose 0.36 mM.

TB: PIPES (free acid) 10 mM, $CaCl_2 \cdot 2H_2O$ 15 mM, KCl 250 mM. Adjust pH to 6.7 with KOH. Add $MnCl_2 \cdot 4H_2O$ 55 mM. Sterilise by filtration.

For transformation of plasmids and ligations in DH5α λpir and M182 strains a different method consisting on treating cells with the chemical TSS[302] was followed. Competent cells prepared with this method must be transformed immediately as they can't be stored. In this case, a saturated culture of the bacteria was diluted 100-fold in LB medium and was grown at 37 ºC until early exponential phase. Afterward, 1 mL aliquots of the culture were prepared in Eppendorf tubes, they were kept in ice for 5 minutes and then centrifuged for 1 minute at maximum speed in a microcentrifuge. Lately, supernatant was discarded, the pellet resuspended in 75 µL of cold LB, the tubes incubated in ice for 5 minutes and finally 75 µL of cold TSS 2x added. The samples were

gently mixed and kept in ice for 5 more minutes and after that the cells were ready for transformation.

TSS 2X: tryptone 0.8 % (w/v), yeast extract 0.5 % (w/v), NaCl 85.5 mM, PEG-8000 25 mM, DMSO 10 % (v/v), $MgSO_4$ 100 mM, pH 6.5.

Heat-shock transformation

For heat-shock transformation between 1-5 µL plasmid or 10 µL of ligation were added to 50 µL of competent cells prepared by the first method or to a complete aliquot prepared by the second method. The samples were kept in ice for 30 minutes and then given a heat-shock at 42 ºC for 45 seconds. Tubes were immediately put in ice and kept there for 5 minutes. Afterwards, 1 mL SOC was added to the samples and they were incubated for 1 hour at 37 ºC and 180 rpm agitation. Last, 50 µL, 100 µL and the remaining volume were plated in LB with the selective antibiotic plates and grown overnight at 37 ºC.

2.2. *Sphingopyxis granuli* TFA transformation by electroporation

Electrocompetent cells preparation

For transformation of plasmids in TFA by electroporation, electrocompetent cells were prepared as follows. TFA was inoculated in 10 mL MML medium with the corresponding antibiotic and grown overnight for approximately 24 hours until saturation. The inoculum was then diluted in 100 mL of the same medium to an OD_{600} of 0.1 and grown at 30 ºC until OD_{600} 0.4-0.5. The culture was then kept in ice for at least 30 min and then centrifuged for 15 min at 5000 rpm and 4 ºC. After that cells were successively washed with 100 mL and 50 mL of Milli-Q water and 2 mL of glycerol 10% in Milli-Q water. Finally, cells were resuspended in 250 µL of glycerol 10% and 40 µL aliquots were stored at -80 ºC.

Electroporation of TFA

In order to electroporate a plasmid in TFA, 1-5 µL plasmid were added to an aliquot of TFA electrocompetent cells, keeping the cells in ice. Mixes were introduced in cold electroporation cuvettes with a 2 mm distance between electrodes and a electric potential difference of 2.5 kV was applied using a Micropulser TM electroporator (BioRad). Afterwards, 1 mL of cold MML with

sorbitol 0.5 M was immediately added to the cuvette, the resuspended cells transferred to an sterile Eppendorf tube and incubated for 1h at 30 ºC and 180 rpm agitation. Dilutions of 10^6-10^9 were plated in non selective plates to calculate viable cells and dilutions 10^1-10^3 and the rest of the sample in selective plates to obtain transformant cells. All plates were incubated at 30 ºC for 4 or 5 days.

3. Nucleic acids manipulation

3.1. DNA handling

3.1.1. Plasmid DNA isolation in *Escherichia coli*

In order to isolate plasmids from *E. coli* DH5α and DH5α λpir two methods were used. The first method was the simplified protocol of alkaline lysis[303] in which the biomass of 1.5-5 ml, depending on the copy number of the plasmid, obtained by centrifugation in a microcentrifuge at 13000 rpm for 1 minute of the cultures, was resuspended in 100 µL of cold miniprep solution I (GTE). Then 200 µL of fresh room temperature miniprep solution II were added and the sample mixed by inversion and incubated in ice for 5 minutes. After this time 150 µL of cold miniprep solution III were added and the sample mixed by inversion and incubated in ice for 5 more minutes. After a 10 minutes centrifugation of the samples at 13000 rpm in a microcentrifuge, 400-450 µL of the supernatant were transferred to a new Eppendorf tube, 450 µL of cold 96% ethanol were added and the sample was mixed and centrifuged for 10 minutes. The supernatant was discarded and the DNA pellet washed with 1 ml of cold ethanol 70%, being centrifuged for 5 minutes in the same conditions. The supernatant was subsequently discarded and the pellet dried by vacuum. Finally, the DNA pellet was resuspended in 30 µL TER 1x and incubated at 37 ºC for 10 minutes so that the RNAses work. Plasmid samples were stored at -20 ºC.

Miniprep solution I (GTE): glucose 50 mM, EDTA 10 M, Tris-HCl 25 mM pH 8.

Miniprep solution II: NaOH 0.2N, SDS 1 % (w/v).

Miniprep solution III: Potassium acetate 3 M adjusting pH at 4.8 with formic acid.

TER: Tris-HCl 10 mM pH 8, EDTA 1 mM, ribonuclease (Roche) 0.02 % (w/v).

The second method, used for the cases in which we needed cleaner and higher quality plasmid DNA, was the use of the commercial kit NucleoSpin® Plasmid (Macherey-Nagel), following the protocol indicated by the manufacturer.

3.1.2. Total DNA isolation in *Sphingopyxis granuli*

Isolation of genomic DNA of TFA was performed from 1.5-5 ml saturated MML cultures of this bacteria using the commercial kit Wizard® Genomic DNA purification (Promega) following the instructions given by the manufacturer.

3.1.3. Polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) were performed using different polymerases depending on the necessity or not of high fidelity in the PCR product. When high fidelity was needed, in DNA fragments for cloning, iPfu Polymerase (Thermo Scientific) and Q5® High-Fidelity DNA Polymerase (NEB) were used. In the cases in which the purpose of the PCR was to check constructions and no sequencing was needed the Taq DNA Polymerase (Promega) and DreamTaq Polymerase (ThermoFisher Scientific) were used instead.

As template for PCRs 25 ng of genomic DNA of TFA for 25 µl reactions was used, especially when the product was intended for cloning purposes. In other cases, 5 µl of a colony of TFA previously resuspended in 20-30 µl water and boiled for 5 minutes could be alternatively used. For PCRs of *E. coli*, the biomass of a colony was directly resuspended in the PCR mix before reaction. Finally, sometimes plasmid DNA was used as control in some PCRs at variable concentration.

For Taq DNA Polymerase and iPfu Polymerase, primers were added to a final concentration of 1 µM, Mg^{2+} to 1.5 mM and dNTPs to 100 µM. For Dreamtaq polymerase primers were added to a final concentration of 1 µM, Mg^{2+} to 2 mM and dNTPs to 200 µM. For Q5 polymerase primers were added to a final

concentration of 0.5 µM, Mg^{2+} to 2 mM and dNTPs to 200 µM. The buffer concentration and the unities of each enzyme used were the recommended for each enzyme. Sometimes a concentration of 5% (v/v) of DMSO was used as it was previously described to make amplification easier when the template G+C content was high[304]. For Q5 enzyme, the High GC Enhancer provided with the enzyme was used alternatively to DMSO.

The PCR reactions were performed in Biometra Tpersonal and Biometra Tgradient thermocyclers, and the programs used were the most adequate for each enzyme, the annealing temperature of each primer and the length of the desired PCR product in each case.

3.1.4. DNA electrophoresis in agarose gels

DNA electrophoresis in agarose gels was performed in order to visualise DNA samples from extractions, PCR and restriction reactions or to purify specific DNA bands. In all the cases low electroendosmosis agarose dissolved in TAE 1X buffer at 0.6-1% (w/v) concentration was used. Electrophoresis samples were mixed with loading buffer 6x so that the final concentration of the buffer was 1x prior to being loaded in the gel. To check the size of the samples, the 1 kb ladder (Invitrogen) and the GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) markers were used and a electric potential difference of 45-135 V was used. Gels were then stained in a 2.5 mg/L ethidium bromide solution and the DNA observed in UV transilluminator (Vilber Lourmat).

Loading Buffer 6x: bromophenol blue 0.05 % (w/v), xylene cyanol 0.05 % (w/v), glycerol 30 % (v/v), TE 50x 2% (v/v).

TAE 1x: Tris-acetic acid 40 mM, EDTA 10 mM, pH 7.7.

3.1.5. DNA fragments purification and cloning

For DNA fragments purification from agarose gels, the corresponding size band was observed by exposition to low intensity UV light and identified using de DNA ladder marker. The band was cut from the gel using a razor and DNA purified from it using the commercial kits GFX® (GE Healthcare) and High Pure

PCR Product Purification (Roche), following the manufacturer instructions. In the case of DNA purification from enzymatic reactions the same kits were used.

For the construction of plasmids, a previously described protocol was used[299]. The restriction enzymes used were from New England Biolab and Roche and the supplied buffer and recommended protocol were followed. Ligation reactions were performed in a final volume of 10 µL. Two different ligases were used during this thesis. For T4 DNA ligase from Roche, kinase buffer was used with ATP at a concentration of 1 mM for cohesive ends and of 0.1 mM for blunt ends. One unit of ligase was added to each reaction and they were incubated for at least 12 hours at 16 ºC. For DNA ligase from NEB, the supplied buffer was used, one unit of ligase was added to the reactions and they were incubated for 2 hours at room temperature.

Kinase buffer 10x: Tris-HCl 0.5 M pH 7.6, Cl_2Mg 0.1 M, DTT 50 mM, BSA 0.05 % (w/v).

- **pMPO704**. TFA *fnrN* gene was amplified by PCR (702 bp in total, 6 bp upstream the ATG) using the primers fnrLHindIIIF and fnrLXbaIR. The resulting product was cut from gel and purified and this product and the plasmid pIZ1016 were digested with the restriction enzymes HindIII and XbaI. The digested plasmid and product were ligated and transformed in *E. coli* DH5α.

- **pMPO705**. TFA *fixK* gene was amplified by PCR (815 bp in total, 67 bp upstream the ATG) using the primers fixKPstIF-2 and fixKXbaIR-2. The resulting product was cut from gel and purified and this product and the plasmid pIZ1016 were digested with the restriction enzymes PstI and XbaI. The digested plasmid and product were ligated and transformed in *E. coli* DH5α.

- **pMPO706**. TFA *fleQ* gene was amplified by PCR (1279 bp in total, 94 bp upstream the ATG) using the primers nifA6 F2 and nifA6 Rv3. The resulting product was cut from gel and purified and this product and the plasmid pSEVA224 were digested with the restriction enzymes

EcoRI and SphI. The digested plasmid and product were ligated and transformed in *E. coli* DH5α.

- **pMPO707**. TFA *fnrN* flanking upstream (915 bp) and downstream (1160 bp) regions were amplified using the primers fnrLF1F, fnrLF1R, fnrLF2F and fnrLF2R respectively. Upstream flanking region was digested with EcoRI and BamHI and downstream flanking region with BamHI and XbaI and were successively cloned in the plasmid pUC18, being the ligation transformed in *E. coli* DH5α. Afterwards, the plasmid was digested with EcoRI and XbaI and the fragment holding the two flanking regions was cloned using these same enzymes in pEMG, being the ligation transformed in *E. coli* DH5α λpir.

- **pMPO708**. TFA *fixK* flanking upstream (1015 bp) and downstream (1196 bp) regions were amplified using the primers fixKF1F, fixKF1R, fixKF2F and fixKF2R respectively. Upstream flanking region was digested with EcoRI and BamHI and downstream flanking region with BamHI and XbaI and were successively cloned in the plasmid pUC18, being the ligation transformed in *E. coli* DH5α. Afterwards, the plasmid was digested with EcoRI and XbaI and the fragment holding the two flanking regions was cloned using these same enzymes in pEMG, being the ligation transformed in *E. coli* DH5α λpir.

- **pMPO709**. TFA *narG* flanking upstream (1269 bp) and downstream (1224 bp) regions were amplified using the primers narGF1F, narGF1R, narGF2F and narGF2R respectively. Upstream flanking region was digested with SacI and BamHI and downstream flanking region with BamHI and XbaI and were successively cloned in the plasmid pUC19, being the ligation transformed in *E. coli* DH5α. Afterwards the plasmid was digested with SacI and XbaI and the fragment holding the two flanking regions was cloned using these same enzymes in pMPO1412, being the ligation transformed in *E. coli* DH5α λpir.

- **pMPO710**. TFA *fleQ* flanking upstream (1080 bp) and downstream (1104 bp) regions were amplified using the primers nifA6F1F,

nifA6F1R, nifA6F2F and nifA6F2R respectively. Upstream flanking region was digested with EcoRI and BamHI and downstream flanking region with BamHI and HindIII and were successively cloned in the plasmid pUC19, being the ligation transformed in *E. coli* DH5α. Afterwards the plasmid was digested with EcoRI and HindIII and the fragment holding the two flanking regions was cloned using these same enzymes in pMPO1412, being the ligation transformed in *E. coli* DH5α λpir.

- **pMPO711**. TFA *ctrA* flanking upstream (1160 bp) and downstream (1184 bp) regions were amplified using the primers ctrA F1 Fw, ctrA F1 Rv, ctrA F2 Fw and ctrA F2 Rv respectively. Upstream flanking region was digested with Acc65I and EcoRI and downstream flanking region with Acc65I and XbaI and were successively cloned in the plasmid pUC19, being the ligation transformed in *E. coli* DH5α. Afterwards the plasmid was digested with EcoRI and XbaI and the fragment holding the two flanking regions was cloned using these same enzymes in pMPO1412, being the ligation transformed in *E. coli* DH5α λpir.

- **pMPO738**. TFA *fliA* flanking upstream (1053 bp) and downstream (986 bp) regions were amplified using the primers fliA1, fliA2, fliA3 and fliA3 respectively. Both flanking regions were cloned in pBluescript SK (+), being the upstream flanking region cloned using the enzymes XhoI and BamHI, while the non-digested PCR of downstream flanking region was cloned in the plasmid digested with EcoRV, and the ligation transformed in *E. coli* DH5α. Afterwards, the fragment holding the two flanking regions was amplified by PCR with primers fliA1 and fliA4 and cloned in pMPO1412 cut with SmaI, being the ligation transformed in *E. coli* DH5α λpir.

3.1.6. DNA fragments sequencing

All clonings were checked by sequencing. Sequencing reactions were performed by Secugen (Madrid) and StabVida (Lisbon). The templates sent were in most of the cases plasmids and PCR products in particular cases and

the primers were specific of the fragment or plasmid sequenced. The concentrations of templates and primers used were the required by each house.

3.1.7. TFA strains construction

For the construction of TFA mutants a variation of a previously described method[296] was used.

For MPO250 and MPO251 mutants, electrocompetent TFA cells were electroporated with pMPO707 and pMPO708 respectively. Cells resistant to kanamycin, which should have the plasmid integrated by homologous recombination, were selected and candidates checked by PCR with the primers aguBF and M13 Rv and fnrLF1F and M13 Rv for pMPO707 and KmFw-pk18 and KmRv-pk18 and FfixKCompF and M13 Rv for pMPO708. Positive candidates of each plasmid were electroporated with pSW-I plasmids and colonies resistant to ampicillin but sensitive to kanamycin, which should have undergone the second recombination event, were selected. Candidates were checked by PCR with the primers pSW-F and pSW-R for the presence of pSW-I plasmid. Positive candidates were grown for several generations without ampicillin and ampicillin sensitive candidates that have lost pSW-I plasmid were selected and the *fnrN* and *fixK* deletions, respectively, checked by PCR with the primers aguBF and fnrLF2R for MPO250 and FFixKcompF and fixKF2R for MPO251 and by Southern blot. For MPO252 mutant, the single mutant MPO251 was electroporated with pMPO707 and the same protocol described above for the single mutants was followed, checking the candidates by PCR with the primers aguBF and fnrLF2R and Southern blot.

For MPO253, MPO254, MPO256 and MPO851 mutants a further variation of the above explained method[289,296] was used. Electrocompetent TFA cells were electroporated with pMPO709, pMPO710, pMPO711 and pMPO738 plasmids respectively and, in this case, cells resistant to kanamycin but sensitive to streptomycin 200 mg/L, which should have the plasmid integrated in the chromosome by homologous recombination, were selected and checked by PCR with the primers rpsl1 Fw and rpsl1 Rv. Electrocompetent cells of a positive candidate of each electroporation were electroporated with pSW-I plasmid and candidates resistant to ampicillin and streptomycin 50 mg/L but

sensitive to kanamycin, which should have undergone the second recombination event, were selected. Candidates were checked by PCR with narGF1F and narG qPCR R primers for MPO253, with nifA6 F2 and nifA6 R2 primers for MPO254, with Comp mut ctrA Fw and Seq F1 ctrA Rv primers for MPO256 and with fliA1 and fliA4 plasmid for MPO851. Positive candidates were grown for several generations without ampicillin and ampicillin sensitive candidates that had lost pSW-I plasmid were selected.

3.1.8. DNA hybridisation (*Southern Blot*)

Mutants MPO250, MPO251 and MPO252 were additionally checked by Southern Blot. For this purpose, probes homologous to the sequence of the fragment that was to be checked were designed. For MPO250 and MPO252 this probe was constructed amplifying the downstream flanking region of *fnrN* with primers fnrLF2F and fnrLF2R and for MPO251 the probe was constructed amplifying also the downstream flanking region of *fixK* with primers fixKF2F and fixKF2R. Then, probes were marked using the random priming method with digoxigenin-dUTP (Roche) following the protocol provided by the manufacturer.

Genomic DNA of each mutant, as well as the wild type strain, the wild type strain with the plasmids pMPO707 and pMPO708 and MPO251 with the plasmid pMPO707 integrated was extracted and 2 µg of each DNA were digested with the corresponding restriction endonucleases. For MPO250 and MPO252 BamHI, EcoRV+SmaI and NcoI enzymes were used while PvuII, AatII and NdeI+SspI were used for MPO251. The restriction fragments of each sample were then separated by electrophoresis in a 0.6% (w/v) agarose gel at 45V and 4 ºC and the electrophoresis gel was subsequently treated as described before[299]. The samples in the gel were depurinated by an incubation in 0.25 M HCl for 20 minutes, then denaturised for 30 minutes in a solution of 1.5 M NaCl and 0.5 M NaOH and finally neutralised by to washes of 15 minutes in a solution of 1 M Tris-HCl and 1.5 M NaCl pH 7.4. DNA was then transferred to a nylon Hybond N membrane (GE Healthcare) by pressure using SSC 10x as transfer buffer. The membrane was dried in the laminar flow cabinet and the DNA fixed in the membrane by crosslinking by exposure to UV light at 254 nm and 700 V for 30 seconds.

The membrane was then pre-hybridisated in the hybridisation furnace Techne® HB-3D with a pre-hybridisation solution previously heated at 65 ºC for at least 10 minutes. The membrane was subsequently incubated overnight for 14-16 hours at 42 ºC with fresh pre-hybridisation solution to which the DNA probe, previously boiled, was added. After this incubation the membrane was washed in SSC 2x with 0.1% (w/v) SDS twice at room temperature for 5 minutes each time and two more times in SSC 0.1x with 0.1% (w/v) SDS at 68 ºC for 15 minutes each time.

All the following steps were performed at room temperature and with stirring. For the detection of the probe, first the membrane was equilibrated in Buffer 1 for 1 minute and then blocked for one hour with 20 ml blocking solution. After that, the anti-DIG-alkaline phosphatase antibody was added to a proportion of 1 µl per 10 ml blocking solution and the membrane incubated for 30 minutes. Then the membrane was washed twice with Tween 20 0.3% in Buffer 1 for 15 minutes each time and equilibrated for 2 minutes in Buffer 3. After that, the membrane was incubated for 5 minutes in Buffer 3 with the luminescent substrate of alkaline phosphatase CSPD® (Roche) at concentration 1:300. Then the membrane was introduced in a hybridisation bag and incubated for 15 minutes at 37 ºC to activate the alkaline phosphatase. Finally the chemo-luminescent images were observed using the image system ChemiDoc™ (BioRad).

Depurination solution: HCl 0.25 M.

Denaturation solution: NaCl 1.5 M, NaOH 0.5 M.

Neutralisation solution: Tris-HCl 1M, NaCl 1.5 M, pH 7.4.

SSC 20x: NaCl 175 g/L, Sodium citrate $\cdot 2H_2O$ 88 g/L, adjust pH to 7 with HCl 1 M.

Pre-hybridisation solution: SSC 5x, N-lauroylsarcosine 0.1% (w/v), blocking reagent 1% (w/v) (Roche), formamide 50% (v/v), salmon sperm DNA 0.05 mg/L. Salmon sperm DNA must be added when the solution is going to be used and it must be boiled beforehand for 10 minutes.

Buffer 1: Maleic acid 100 mM, NaCl 150 mM, pH 7.5.

Buffer 3: NaCl 0.1 M, $MgCl_2$ 0.05 M, Tris-HCl 1M, pH 9.5.

Blocking solution: Blocking reagent (Roche) 10% (w/v) in Buffer 1.

3.2. RNA handling

3.2.1. RNA isolation in *Sphingopyxis granuli*

Pellets for RNA isolation were obtained by centrifugation of 10 ml of cultures grown to an OD_{600} of 0.7-0.8 or the highest volume possible of cultures grown to lower optical densities when the volume necessary to achieve the same biomass was too high. Cultures were centrifuged for 3.5 minutes at 16100 g and 4ºC, supernatants were discarded and pellets immediately frozen with liquid N_2.

For RNA isolation from frozen cell pellets, samples must be kept all the time, unless specified, in ice and the work place, pipettes must be cleaned beforehand with NaOH 0.2 M and nuclease-free filter pipette tips used.

First, cell pellets were carefully resuspended in 1.5 ml of Tripure Isolation Reagent (Tri Reagent LS, Molecular Research Centre) and then incubated for 10 minutes at 60 ºC, mixing the samples by inversion every 2-3 minutes in order to lysate the cells. Then, samples were centrifuged for 15 minutes at 16100 g and 4 ºC to precipitate the cell debris and the supernatant was transferred to Phase Lock Gel™ (5PRIME) tubes previously centrifuged for 2 minutes at 16100 g. Each sample must be distributed in two Phase Lock Gel tubes, as they only admit 750 µl of sample. Afterwards, 200 µl of chloroform were added to each tube, the samples were mixed by inversion, kept in ice for 15 minutes and then centrifuged for 15 minutes at room temperature and 16100 g. For aqueous phase extraction, 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added to each tube and they were centrifuged for 5 minutes at 16100 g and 4 ºC. Aqueous phase was then transferred to a new tube, joining the two fractions of each sample again, and 1 volume of cold isopropanol was added to the samples, which were then mixed by inversion and kept in ice for 10 minutes. Tubes were centrifuged for 10 minutes at 16100 g and 4 ºC and the supernatant discarded. Pellets were then washed with 1 ml of cold ethanol 70% (v/v) and

centrifuged again for 5 minutes at 16100 g and 4 ºC. Last, supernatant was discarded and pellets were dried in the laminar flow cabinet and then resuspended in 150 µl of water treated with diethyl pyrocarbonate, a compound that inactivates RNAses (DEPC water).

In order to degrade the DNA that could remain in the ARN samples, they were treated with DNAse I, using the kit DNA free (Ambion) following the instructions of the manufacturer and the purified with the RNAeasy minikit (Quiagen) and eluted in 30-50 µl H_2O DEPC, depending on the RNA yield. Finally, the complete absence of DNA was checked by PCR using the primers phaC1 and phaC4, that are homologous to a region of TFA DNA.

3.2.2. RNA electrophoresis

Before and after treatment with DNAse, RNA samples were visualised by electrophoresis to check their quality and concentration. Electrophoresis gels for ARN were prepared with low electroendosmosis agarose disolved in TAE 1x buffer at 1% (w/v) concentration. Ethidium bromide was directly added to the gel, adding 3 µl of ethidium bromide 10 mg/ml to 40 ml agarose gel before polymerisation. For visualisation, 1 µl of each ARN sample was mixed with loading buffer 6x (w/v), completing the volume with DEPC water so that the final concentration of the loading buffer was 1x. To check the size of the samples, the 1 kb ladder (Invitrogen) and the GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) markers were used and the samples were run for 13-16 minutes at 135 V. RNA was observed in UV transilluminator (Vilber Lourmat).

Loading Buffer 6x: bromophenol blue 0.05 % (w/v), xylene cyanol 0.05 % (w/v), glycerol 30 % (v/v), TE 50x 2% (v/v).

TAE 1x: Tris-acetic acid 40 mM, EDTA 10 mM, pH 7.7.

3.2.3. RNA retrotranscription and cDNA synthesis

For cDNA synthesis, 2 µg of RNA were added to each PCR reaction as template and 3 PCR reactions were performed of each sample in order to obtain higher yields of cDNA. Reverse transcription was performed using the

High capacity cDNA reverse transcription kit (Thermo Fisher Scientific) following the instructions of the manufacturer.

After the retrotranscription, the three PCRs of each samples were mixed and then cleaned using the QIAquick PCR Purification Kit (QIAGEN) following the protocol given by the manufacturer and eluting the samples in 30-50 µl DEPC water.

4. Nucleic acids quantification

For quantification of double strand DNA, including plasmid and genomic DNA, absorbance at 260 nm was measured with NanoDrop ND-1000, NanoDrop One and NanoDrop 2000 spectrophotometers (Thermo Scientific) using an extinction coefficient of 50 ng cm μL^{-1}. For single strand DNA, cDNA in our case, quantification was performed the same way but using an extinction coefficient of 33 ng cm μL^{-1}. Finally, for RNA quantification the extinction coefficient used was 40 ng cm μL^{-1}.

5. Genetic expression analyses

5.1. Real time quantitative PCR (RT-qPCR)

Prior cDNA synthesis, RNA from three biological replicates of each sample were mixed so that that the same RNA quantity of each replicate was added to the mix. For RT-qPCR 10 ng of TFA cDNA per reaction were used for the samples and 25 ng, 2.5 ng, 0.25 ng, 0.025 ng and 0.0025 ng of TFA genomic DNA were used per reaction for the standard curve. Four technical replicates of each sample, the standard curve and the negative control without nucleic acids were carried. All the qPCR primers were designed with a melting temperature of 58-62 ºC and amplified a region of 50-150 nucleotides. RT-qPCRs were performed using two different protocols. In the first one[305], SensiFast™ SYBR® Hi-Rox Kit (Bioline) was used, following the instructions and protocol provided by the manufacturer, the samples were loaded in MicroAmp® Optical 96-Well Reaction Plates (Applied Biosytems) and the RT-qPCR performed in ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). In the second one, FastGene® IC Green 2x qPCR Universal Mix (Nippon Genetics) kit was used, following the instructions and protocol provided by the manufacturer,

the samples were loaded in PCR-plates, 96x0.2ml, full skirted, natural (Nerbeplus) and the RT-qPCR performed in CFX Connect Real-Time PCR Detection System (Bio-Rad).

5.2. High-throughput RNA sequencing by dRNA-seq

For dRNA-seq, RNA from three biological replicates of each sample were mixed so that that the same RNA quantity of each replicate was added to the mix. RNA samples were sent to the aScidea Headquartes (Barcelona, Spain) for library preparation and RNA sequencing. The cDNA libraries were sequenced using an Illumina HiSeq2000 machine[289].

The bioinformatic analysis of the dRNA-seq data was performed by aScidea. Quality of the reads obtained by HiSeq200 sequencing was checked with FastQC software (http:/www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) and pre-processing of the reads was carried out with fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and aScidea specific perl scripts property of aScidea (http://www.ascidea.com) in order to filter regions of low quality. Adaptors were trimmed and low quality bases at the ends of sequences and reads with undetermined bases of with 80 % of their bases with less than 20 % quality score. Raw ends which passed the quality filter threshold were mapped using Bowtie2 2.0.6 [306]. The reference genome and genomic annotations used were obtained from NCBI open databases (https://www.ncbi.nlm.nih.gov/nuccore/CP012199.1)[10].

For the RNA-seq of the WT strain in aerobic and anaerobic conditions, in aerobic conditions with tetralin as carbon source and in aerobic conditions in the presence of NO, the inner distance between mate pairs used was 50 bp and default values of the rest of parameters were taken. Gene level counts were calculated and FPKM normalised using Cufflinks 2.0.2 software[307] and differential transcript expression was then computed using Cuffduff. The free statistical language R was used for the main statistical analyses and the libraries developed for data analyses were performed by the Bioconductor Project (www.bioconductor.org)[308]. FPKM values were used to normalise and qualify the gene expression level and genes differentially expressed more than

3-fold between the different conditions studied were selected for further analysis.

For the RNA-seq of the Δ*fnr* MPO252 and Δ*narG* MPO253 mutants, the protocol followed was different. The previous analysis resulted in a lists of genes from higher to lower by their expression values. Because the process of statistical testing involved one test per gene at a time, resulting p-values were adjusted using Benjamini and Hochberg method[309] to obtain an strict control of the False Discovery Rate (FDR). FeatureCounts from SubRead package in quantification mode was used to obtain transcript and isoform estimates. DESeq2 was used to assess differential expression, and genes with Log2FC > 1 and Log2FC < -1 (Log2 Fold-Change) and a p value < 0.05 were selected as genes differentially expressed (DEG). Genes Differentially expressed more than 3-fold between the different conditions studied were selected for further analysis.

Genes were clustered according to the Cluster of Orthologous Groups of proteins (COGs) broad classification[310].

6. Protein detection by inmunodetection (Western Blot)

Cell extracts preparation

Cell extracts for western blot were prepared by resuspending biomass from TFA cells from plates and from pellets of centrifuged liquid cultures in different media and conditions. Then 200 µl of these samples were mixed with 100 µl of Laemmli Buffer 3x and boiled for 5 minutes at 100 ºC. These samples were stored at -20 ºC.

Laemmli Buffer 3x: 180 mM Tris-Cl pH 6.8, 6% SDS, 30% glycerol, 15% β-mercaptoethanol, 0.03% bromophenol blue.

SDS-PAGE electrophoresis

To separate proteins from the crude protein extracts, an electrophoresis in acrylamide gel 10% and 1 mm thickness of the samples was performed. The gels were prepared using the kit TGX FastCast™ Acrylamide gels (BioRad) following the instructions of the manufacturer.

Materials and methods

Prior to electrophoresis, the extracts had to be unfrozen and heated for 1-2 minutes at 100 ºC to facilitate loading. Different volumes of extract, depending on their protein concentration, were loaded in the gel and Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific) was used as marker. Gels were run in a Criterion™ Vertical Electrophoresis Cell (BioRad) at 150 V for around 1 hour in .

After electrophoresis, gels were washed for 3 times with Milli-Q water and the stained with QC Colloidal Coomassie Stain (BioRad) following the manufacturer instructions. Gels were then visualised using the image system ChemiDoc™ (BioRad) and protein concentrations were adjusted, when necessary, so that all the samples had similar concentrations.

Transference to membrane and immunodetection

For western blots, the same SDS-PAGE gels were used as for the coomassie blue staining, but without this staining. After electrophoresis, proteins were transferred to membrane using the Trans-blot Turbo Transfer Packs and the Trans-Blot® Turbo™ Transfer System (BioRad) at 25 V using the Turbo protocol.

All the next steps were carried out at room temperature and with stirring. After transfer, the membrane was incubated for 30 minutes in 5% skim milk in PBS-T so that the milk would block the membrane. The membrane was incubated for 1 hour in 2% skim milk in PBS-T with the primary antibody and was then washed twice in 2% skim milk in PBS-T, for 5-10 minutes each time. Afterwards, the membrane was incubated for 1 hour in 2% skim milk in PBS-T with the secondary antibody and the membrane was washed twice in 2% milk in PBS-T for 5-10 minutes each time.

For visualisation, the membrane was treated with the Clarity Western ECL Substrate Kit (BioRad) following the instructions of the manufacturer. Membrane was then visualised using the image system ChemiDoc™ (BioRad).

PBS-T: 0.05 % (v/v) Tween-20 in 1x PBS.

PBS 1x: NaCl 0.137M, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM

7. Nitrite and nitrate measurements

Nitrite and nitrate concentrations in the culture media were measured using a previously described method[311]. All nitrite and nitrate determination was always done in minimal medium, as some components of the rich media could interfere with measurements.

For nitrite determination, first of all cells were removed by centrifugation and later filtration of the cultures. The resulting medium was diluted as required and 300 µl of it was mixed with 300 µl of a saturated solution of sulphanilic acid prepared in 20 % (v/v) of HCl and 300 µl of aqueous solution of 0.2 % (w/v) N-(1-naphthyl)-ethylendiamine dihydrochloride. After a 15 minutes incubation at room temperature, absorbance at 540 nm was measured and nitrite concentration calculated using a standard curve prepared with known concentrations of sodium nitrite.

For nitrate determination cultures were also centrifuged and filtered, and nitrite completely removed from the medium by overnight incubation after addition of amidosulphonic acid to a final concentration of 1 % (w/v). Nitrite must be completely removed from the medium because it can interfere with nitrate determination, so nitrite measurement method was applied to the treated samples for reassurance. After nitrite removal, 100 µl of the sample, diluted as required, were mixed with 800 µl an H_2SO_4:H_3PO_4 solution (1:1, v/v) and 100 µl of a fresh solution of 2,6-dimethylphenol (0.12 %, w/v, in concentrated acetic acid). After 20 minutes at room temperature, absorbance at 324 nm was measured and nitrate concentration determined using a standard curve prepared with sodium nitrate.

8. Swimming assays

For TFA swimming assays, 0.3% agar MML rich medium plates and mineral medium plates with 40 mM BHB and streptomycin were prepared and left for 4 hours at room temperature to polymerise. Between 1 and 3 colonies of *S. granuli* TFA and its mutants were punctured in each plate inside the agar and plates were incubated upside up at 30 ºC. For swimming assays in anaerobic conditions, sodium nitrate 20 mM was additionally added to the medium before

polymerisation and the plates were incubated inside anaerobiosis jars where anaerobiosis was reached was reached by the use of Anaerocult A reactive (Merk) and checked by Microbiology Anaerotest indicator (Merck).

During these assays, “bubbles” of faster swimming biomass were observed in some of the swimming circles. Biomass from these bubbles was taken and used to perform new swimming assays in minimal medium and in rich medium. Biomass from the external part of the larger swimming halos was streaked in MML plates that were incubated at 30 ºC for 4 days and mutant MPO255 was isolated.

9. Electron microscopy

For electron microscopy liquid and solid cultures in rich and minimal medium of TFA and MPO254 were used. In the case of cell growing in plates, biomass was resuspended in Milli-Q water. Cooper grids were then put in contact with the liquid cultures or the resuspended solid cultures so that the cells became adhered to them.

For negative staining, cooper grids were carbon activated incubated for 10-30 minutes with 10 µL of vesicle solution and washed three times with PBS (ThermoFisher Scientific). The solution was fixed on the grids by incubating them for 10 minutes in glutaraldehyde (Sigma-Aldrich) 1% in PBS and then washed again twice with PBS and four times with Milli-Q water. The grids were subsequently briefly rinsed with methylcellulose/uranyl acetate pH 4 (provided by the cell microscopy centre) and incubated for 5 minutes with the same solution on ice. Grids were then air-dried and samples were visualised on a Tecnai-12 electron microscope (FEI).

10. Bioinformatic tools

Sequence alignments of TFA genes among them and with genes belonging to other bacteria were performed using Clustal Omega. For sequence alignments of proteins Uniprot webpage align tool was used. For the searching of protein motifs in TFA proteins Pfam online database was used.

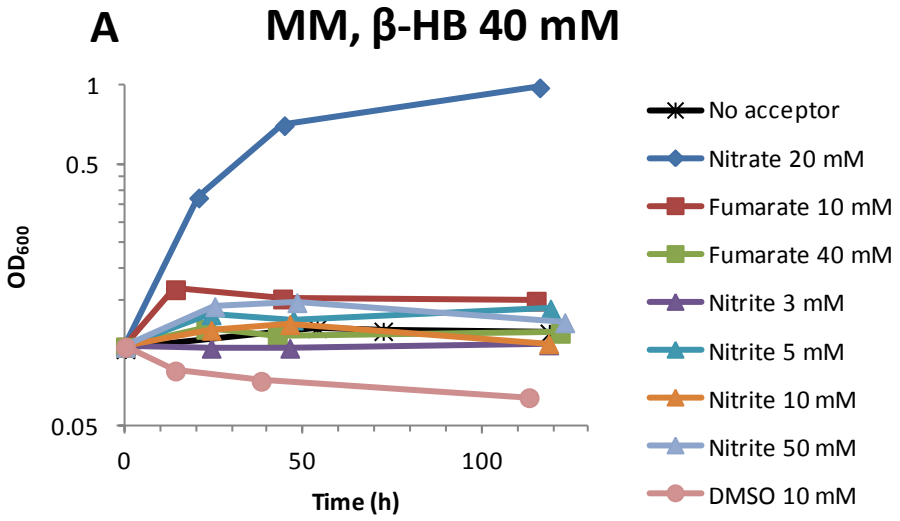
In order to find conserved motifs that could be the recognition sites of Fnr proteins in TFA, the region upstream of selected genes was analysed using the online tool MEME (Multiple Em for Motif Elicitation). First the upstream region of genes found in the dRNA-seq analysis to be 4-fold more induced in MPO253 than in MPO252 was introduced in MEME. This region comprised 400 nucleotides upstream of the ATG and 10 nucleotides downstream the ATG. The tool provided a consensus sequence that was then compared to the previously used genes using the online tool FIMO (Find Individual Motif Occurences) to check which of these genes showed the consensus sequence found. Then, this new selection of genes was analysed again using MEME, obtaining a more curated consensus sequence. The presence of this sequence was checked in the upstream region of the genes 4-fold more induced in MPO253 than in MPO252, suspected to be regulated by Fnr in TFA, to define Fnr operon.

RESULTS

1. Anaerobic growth of *Sphingopyxis granuli* TFA in different media and with alternative final electron acceptors

1.1. Anaerobic incubation of TFA with different final electron acceptors

Sequencing and bioinformatic analysis of the genome of *Sphingopyxis granuli* strain TFA showed that TFA has in its genome the *nar* genes (*narUGHJInifMmoaADEBCmoeA* operon), the genes to respire nitrate to nitrite, but didn't show genes for respiration of other final electron acceptors, except for a putative *cysJI2* operon that might be involved in sulphite respiration or detoxification[10]. However, taking into account the presence of a high number of uncharacterised genes in TFA annotation, we decided to check whether TFA grew in anaerobic conditions using several of the most commonly used final electron acceptors, in addition to nitrate, being them fumarate, nitrite and DMSO. Different concentrations of these final electron acceptors were tested both in mineral and rich media, starting at an OD_{600} of 0.1 and OD_{600} of the cultures was followed over time. As expected, results showed that TFA was only able to grow when nitrate was used as final electron acceptor (Figure 13). Moreover, we have seen that TFA was unable to grow in rich medium MML when no final electron acceptor was added, thus suggesting that this bacterium is unable to ferment (Figure 13B).



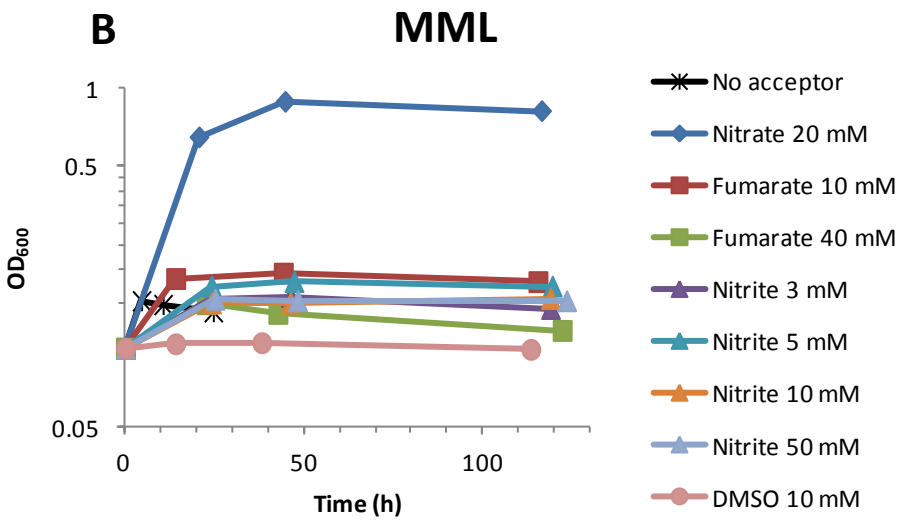


Figure 13. TFA growth in anaerobic conditions with different final electron acceptors. TFA growth in anaerobic conditions was tested without final electron acceptor (cross), with nitrate (diamonds) 20 mM, fumarate (squares) 10 and 40 mM, nitrite (triangles) 3, 5, 10 and 50 mM and DMSO (circles) 10 mM as final electron acceptors in mineral (A) and rich (B) media.

1.2. Anaerobic growth of TFA in rich and minimal medium with nitrate

In order to characterise anaerobic growth of TFA using nitrate as the final electron acceptor, we performed growth curves of this bacterium in anaerobic conditions with nitrate 20 mM in rich MML medium or mineral medium with β-HB as the carbon source, and compared them to aerobic conditions. The preinocula came from the same media in each case, always without nitrate and in aerobic conditions (Figure 14). Growth was successfully observed in all the conditions and media, reaching the cultures higher OD_{600} in mineral medium than in MML, both in aerobic and anaerobic conditions.

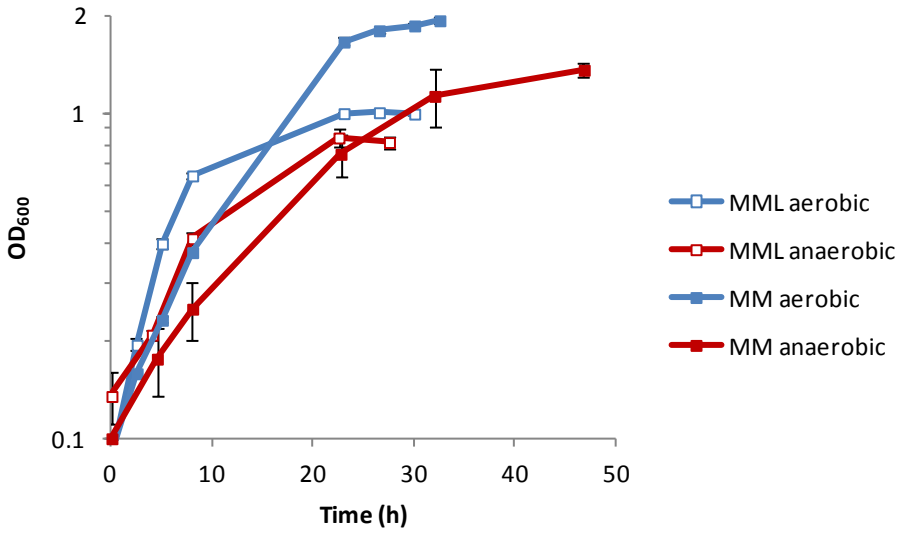


Figure 14. Aerobic and anaerobic growth curves of TFA. TFA growth, represented by Optical density at 600 nm (OD_{600}), in aerobic (blue) and anaerobic (red) conditions in rich MML medium (empty squares) and mineral medium with β-HB 40 mM (filled squares). In anaerobic conditions nitrate 20 mM was added as final electron acceptor. Graphics represent the mean ± SD of at least 3 biological replicates.

From the data of the OD_{600} evolution over time of all the replicates, generation times and growth rates of TFA in all the conditions were also calculated (Table 6). Regarding oxygen availability, growth rates were higher and generation times lower in aerobic conditions compared to anaerobic conditions in both media. Regarding the media composition, growth rates were higher and generation times lower in rich medium compared to mineral medium in both conditions.

	Medium	Generation time (h)	Growth rate (h^{-1})
Aerobic	MML	2.2±0.1	0.31±0.02
	MM, β-HB 40 mM	3.89±0.05	0.178±0.002
Anaerobic	MML, NO_3^- 20 mM	4.8±0.8	0.14±0.02
	MM, β-HB 40 mM, NO_3^- 20 mM	6.3±0.7	0.11±0.01

Table 6. Generation times and growth rates of TFA in aerobic and anaerobic conditions. Generation times and growth rates of TFA growing in aerobic and anaerobic conditions in rich MML medium and mineral medium with β-HB 40 mM. In anaerobic conditions nitrate 20 mM was added as the final electron acceptor. The table shows the mean ± SD of at least 3 biological replicates.

Furthermore, in order to determine if the nitrate reductase genes found were responsible for nitrate respiration and anaerobic growth in TFA, a deletion mutant of one of the genes of the operon, *narG*, putatively coding for a subunit of the nitrate reductase enzyme[58], was constructed and its anaerobic growth tested. The deletion of this gene was almost complete, of 3624 bp out of 3744 bp, and no marker was introduced, as fully explained in Materials and Methods. We observed that this mutant, MPO253, as expected, was unable to grow anaerobically in mineral medium with β-HB 40 mM and nitrate 20 mM (Figure 15). This suggests that this operon codes for the actual *nar* genes responsible for nitrate anaerobic respiration in TFA.

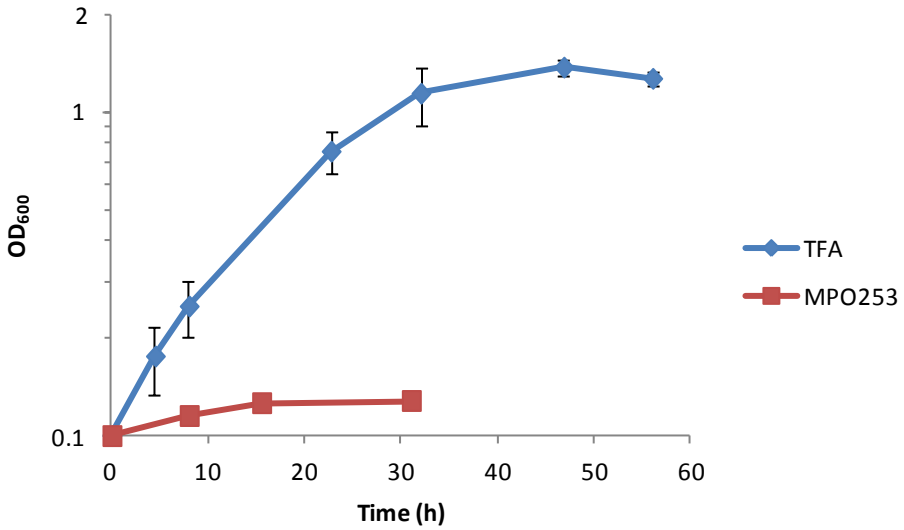


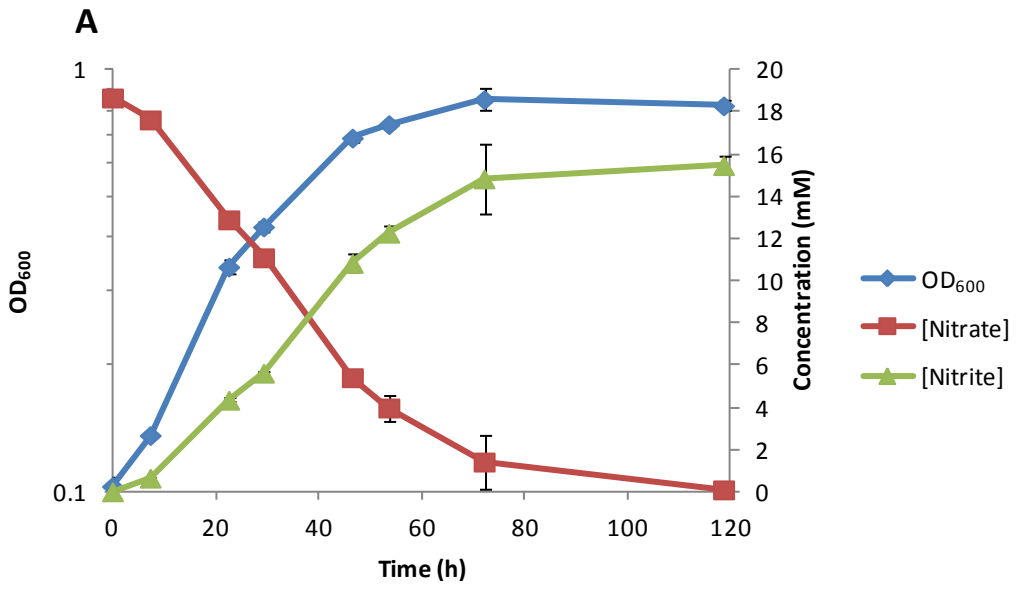
Figure 15. Anaerobic growth of Δ*narG* mutant. Growth, represented by Optical density at 600 nm (OD_{600}), of TFA (blue) and Δ*narG* mutant MPO253 (red) in mineral medium with β-HB 40 mM and with nitrate 20 mM as final electron acceptor. Graphics represent the mean ± SD of at least 3 biological replicates.

1.3. Nitrate consumption and nitrite production

Knowing that TFA was able to grow anaerobically when nitrate was added in the culture medium, we wanted to measure nitrate consumption and nitrite production over anaerobic growth. For this purpose we measured nitrite and nitrate concentrations in the culture medium of TFA growing in anaerobic conditions in mineral medium with nitrate 20 mM. The preinocula to inoculate these cultures came from rich MML medium. Results show that nitrate was completely consumed and nitrite accumulated in approximately stoichiometric

amounts, reaching a final concentration of around 16 mM (Figure 16A). This suggests that TFA is respiring nitrate to nitrite but is not able to respire nitrite, although the 4 mM difference in stoichiometry indicates that part of the nitrate or the nitrite is being lost by a different process. One possible reason for this difference could be the production of nitric oxide during nitrate respiration by the nitrate reductase, as it has been reported previously in *Salmonella* and other bacteria[102,103].

In order to determine if growth was limited by nitrate concentration, as it is completely consumed, we repeated the experiment of Figure 16A, but using the double amount of nitrate, 40 mM (Figure 16B). In this case the final OD_{600} did not increase dramatically, reaching a maximum of around 0.95, similarly to the 0.85 with half the concentration of nitrate, but nitrate was not completely consumed. These results suggest that 20 mM nitrate is not a limiting factor for anaerobic growth. We also considered the possibility that nitrite could be toxic for TFA, as described in other bacteria[101,312] and that nitrite accumulation may be limiting the growth. In order to check this we repeated the assay in Figure 16A but adding an initial concentration of nitrite 6 mM (Figure 16C). This time we observed that the final OD_{600} was much lower, around 0.58 in contrast to around 0.85, and that not all the nitrate was consumed, being the final nitrite concentration reached very similar to Figure 16A. These results suggest that nitrite could be toxic for TFA in anaerobic conditions, thus preventing growth when accumulated in the cultures at a 18-20 mM concentration.



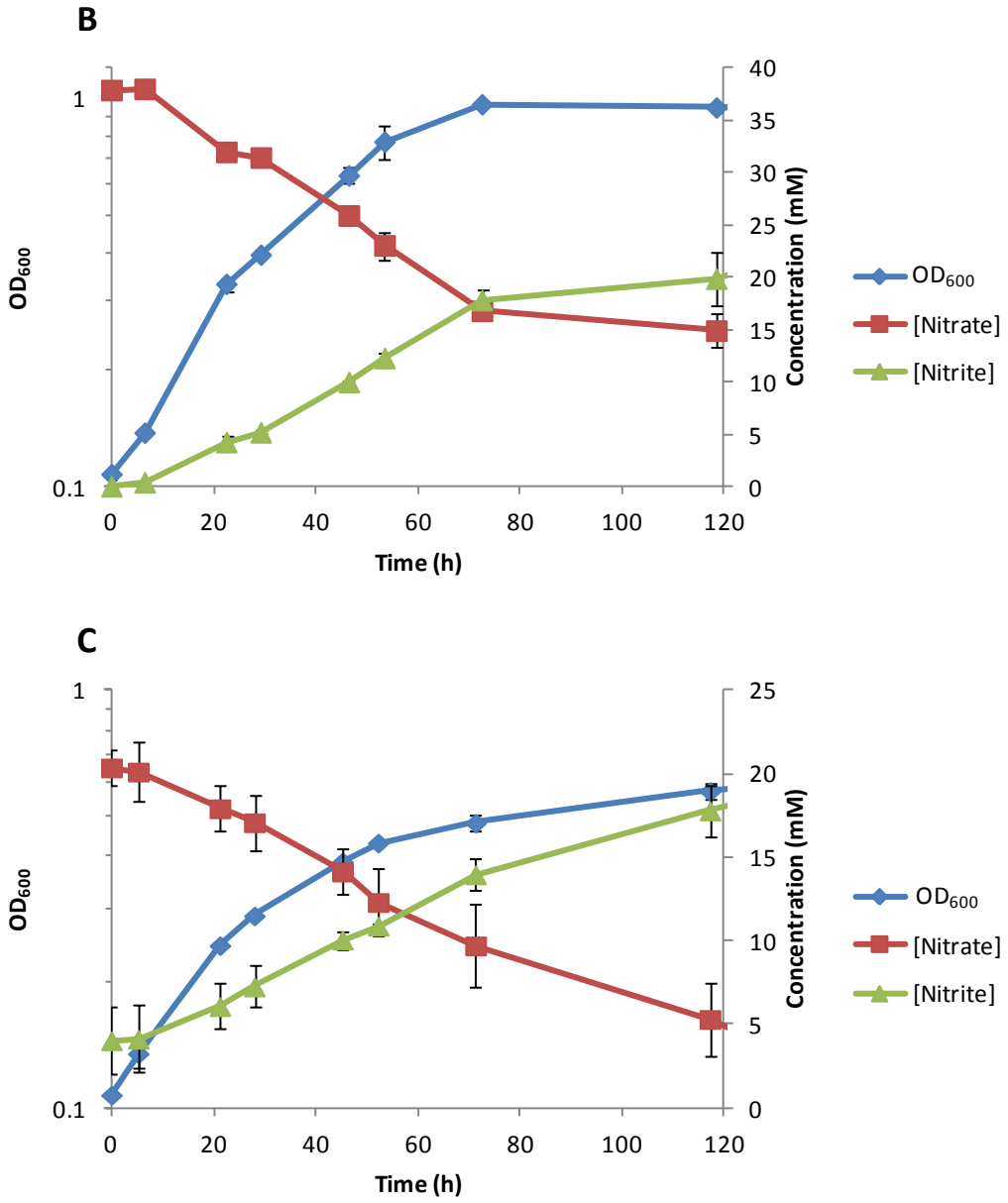


Figure 16. Nitrite and nitrate concentrations over time in cultures in different media. Nitrate (red line) and nitrite (green line) concentrations in the culture medium over an anaerobic growth curve (OD_{600} shown in blue). Mineral media with β-HB 40 mM and with nitrate 20 mM (A), nitrate 40 mM (B) and nitrate 20 mM and nitrite 6 mM (C) were used. Graphics represent the mean ± SD of 2 biological replicates.

Generation times and growth rates in these three conditions were also calculated (Table 7). Generation time was higher and growth rate lower when nitrate 40 mM was used instead of 20 mM. The highest generation time and lowest growth rate was reached at a 20 mM of nitrate when an initial concentration of nitrite was also added to the culture medium. This confirms that the presence of nitrite has a detrimental effect of TFA growth.

Medium	Generation time (h)	Growth rate (h^{-1})
MM, β-HB 40 mM, NO_3^- 20 mM	13.6±0.0	0.05±0.00
MM, β-HB 40 mM, NO_3^- 40 mM	14.7±0.3	0.047±0.001
MM, β-HB 40 mM, NO_3^- 20 mM NO_2^- 6 mM	19.4±0.5	0.035±0.001

Table 7. Generation times and growth rates of TFA in anaerobiosis with different concentrations of nitrate and nitrite. Generation times and growth rates of TFA growing in anaerobic conditions in mineral medium with β-HB 40 mM, coming from MML preinocula, with different initial concentrations of nitrite and nitrate. The table shows the mean ± SD of 2 biological replicates.

2. Response of TFA to anaerobic conditions

In order to study the global response of TFA to anaerobic conditions, we performed a dRNA-seq analysis of this strain comparing growth with β-HB as carbon source in aerobic and anaerobic conditions respiring nitrate. Moreover, we wanted to determine which genes were directly regulated by anaerobiosis, which ones by other conditions related to limited growth[313], such as slow growth, or carbon source or energy limitation, and which ones by other kind of stress factors. For that purpose we performed other transcriptomic analyses to compare with the results obtained in aerobiosis and anaerobiosis, being those aerobic growth with tetralin as the only carbon source and energy source and aerobic response to NO while growing on β-HB. In all the cases, cultures were grown in minimal media, with tetralin in the gas phase as carbon source in the case of aerobic growth with tetralin, or with β-HB 40 mM as carbon source in the rest of the cases. For anaerobic conditions, nitrate 20 mM was added as final electron acceptor and the cultures were incubated in standing stoppered bottles filled to the top to achieve the anaerobic conditions. All the cultures were grown to the exponential phase, OD_{600} of 0.7-0.8, and then cell pellets were taken and frozen for RNA extraction. In the case of the aerobic response to NO, once OD_{600} 0.7-0.8 was reached, DETA-NO 4 mM was added to the cultures, which were incubated for an additional hour, and then pellets were taken and frozen for RNA extraction.

The results of these dRNA-seqs can be found in the Appendix. Results showed a total of 586 genes differentially transcribed at least 3-fold between aerobic and anaerobic conditions, 366 of them induced while 220 repressed in

anaerobiosis. The differentially expressed genes belong to different functional categories, the number of differentially transcribed genes belonging to each COG functional category is indicated in Figure 17, although many differentially transcribed genes are not listed in any of the categories as they code for uncharacterised proteins or belong to the "general function prediction only" or to the "function unknown" categories. Nonetheless, the analysis of the differential expression of genes of these different functional categories gave us a general insight in the response of this bacterium to anaerobic conditions.

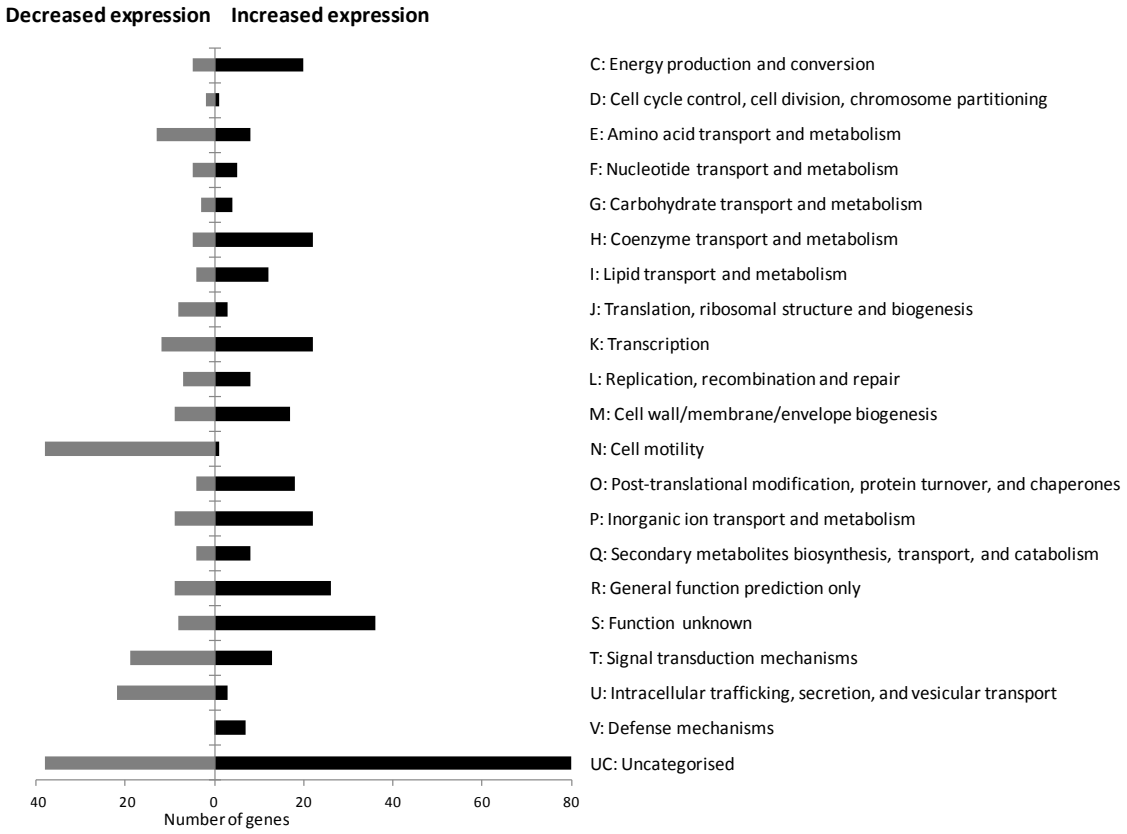


Figure 17. Distribution of anaerobically regulated genes of TFA in COG categories. Genes represented here showed a differential expression of at least 3-fold between growth in anaerobic and aerobic conditions on β-HB.

As we can see in Figure 17, the number of genes differentially transcribed in anaerobic conditions belonging to the COG categories E, F and G was not very high, suggesting that the metabolism of TFA is not greatly affected in anaerobic conditions. Moreover, the fold change of most of the affected genes of this category was low, 6-fold at most, which also supports this idea.

Many genes involved in energy production and conversion (C), however, were affected in anaerobic conditions, although most of them code for electron transfer chains, like *cyo*, *cyd* and *nar* genes.

We have observed that most genes coding for ribosomal proteins and related factors, such as elongation factors G, Tu, Ts and EF-4, and the ribosome recycling factor *frr,* were repressed between 2 and 4-fold. This differential expression in anaerobic conditions of ribosomal proteins could be a consequence not of anaerobiosis but of the slower growth rate, as it also happened even to a greater extent in other slow growth conditions such as aerobic growth on tetralin as carbon and energy source. Surprisingly, in contrast with this, one of the three genes that codes for a translational initiation factor, factor-1 enconded by *infA*, was induced in these conditions. Also, the contiguous genes that code for the ribosomal silencing factor RsfS and the 23S rRNA methyltransferase were specifically induced in anaerobiosis 4-fold, not being induced in aerobic slow growth conditions, which suggests that ribosomes might be modified as an adaptation to anaerobic conditions.

A significant number of genes involved in lipid transport and metabolism (COG category I) and of cell wall/membrane/envelope biogenesis (M) were differentially expressed in anaerobiosis, including many transporters, polysaccharide biosynthesis proteins and cell wall modification proteins such as lytic transglycosylases and 2 of the 3 cellulose biosynthesis proteins. This fact made us think that the lipid, saccharide and proteinic composition of the cell envelope could be very different in aerobic and anaerobic conditions. Furthermore, the differences of membrane-associated proteins should be even greater as the majority of the differentially expressed genes of P and V categories were components of transport systems.

A great amount of motility genes (N), all of them flagellar, pilin and chemotaxis genes, were repressed in anaerobic conditions, as also were other flagellar and pili genes assigned to the intracellular trafficking, secretion, and vesicular transport COG category (U), and other chemotaxis genes assigned to the signal transduction category (T).

Results

As for post-translational modifications, proteins turnover and chaperones category (O), two proteases and a chaperone were induced in anaerobiosis, but most of the genes of this category that were induced in these conditions code for proteins related to stress/detoxification/repair such as peroxiredoxins, thioredoxins, methionine sulphoxide reductase MsrB, sulphide:quinone reductase, the SufABCD proteins involved in Fe-S clusters biogenesis, the DNA repair protein RadA and one Glutathione S-transferase-like protein.

Finally, 38 genes coding for transcription factors such as sigma factors, sensors and transcriptional regulators, belonging to the transcription (K) and signal transduction (T) categories, were also differentially regulated 3 or more fold and are probably responsible for the different regulation levels of the global response of TFA to anaerobic conditions. Interestingly, SGRAN_1132 that codes for the global translational repressor CsrA (RsmA) was induced 6-fold in anaerobiosis.

In order to validate these results and to study the induction kinetics of genes belonging to some of these different functional categories over time, a number of genes were selected and RT-qPCR experiments performed along aerobic and anaerobic growth curves.

2.1. Respiratory electron transfer chains

Our dRNA-seq results show that the conventional aerobic cytochrome *c* oxidase aa_3 was affected less than two-fold. However, TFA has annotated in its genome up to 5 alternative terminal electron acceptors: the cytochrome *c* oxidase cbb_3 (coded by *cco* genes), quinol oxidases bo_3 and *bd* (*cyo* and *cyd* genes respectively), the alternative oxidase *aox* that transfer electrons to oxygen, and the nitrate reductase.

The genes for the putative nitrite/nitrate antiport protein, the nitrate reductase components and the proteins involved in molibdenum cofactor biogenesis, are all clustered in the operon *narGHJInifMmoaADEBCmoeA*, being all of them very close or even overlapped, which suggest that many of them could be translationally coupled. In our dRNA-seq these genes appeared induced from 20 to 40-fold in anaerobic conditions as compared to aerobic conditions. The

genes *mobA* and *moeB*, putatively involved in the nitrate reductase molibdenum cofactor biosynthesis, are located outside of this operon and were also induced anaerobically 8 and 20-fold respectively.

Induction kinetics showed that *narG* gene was induced in anaerobic conditions in the WT strain up to 200-fold in the first 6 hours, and then its expression decreased progressively (Figure 18A). In fact, at the time in which the samples for the dRNA-seq were taken, the induction had already decreased drastically. In the Δ*narG* mutant MPO253 this gene was only induced 6-fold, whilst its expression in anaerobic conditions without nitrate in the medium was even repressed 10-fold. This suggests that not only is the presence of nitrate needed as described in other bacteria[14], but also appropriate anaerobic respiration is essential for full induction.

The quinol oxidase bo_3 is encoded by the *cyoABCDsurF1regBA* operon, in which the genes are also very close or even overlapped with each other, being translation coupling between them very likely. In the RNA-seq, this operon appeared induced in anaerobic conditions, though less than the *nar* operon. However, it is surprising that in *E. coli* this operon is regulated in the opposite way, being repressed in anaerobic conditions and induced in aerobiosis[314]. RT-qPCR assays showed that *cyoC* was induced up to 35-fold after 6 hours of anaerobic growth and then its mRNA level was maintained (Figure 18B). In anaerobic conditions without nitrate in the WT strain and in anaerobic conditions with nitrate in the Δ*narG* MPO253 mutant this gene was actually repressed 3 fold, thus suggesting that nitrate respiration is necessary for its induction. According to the dRNA-seq data, this operon was also induced to some extent, between 2.4- and 8.9-fold, in aerobic conditions when nitric oxide was added to the culture medium. However, the RT-qPCR assays showed that the addition of the same concentration of NO in anaerobic conditions did not induce the expression of *cyoC*; in fact, it was repressed 3-fold.

The quinol oxidase *bd* is encoded by the divergent operons *cydAB* and *cydDC*, together with other genes coding for membrane or unknown proteins. All of them were induced between 3.3- and 9.7-fold in anaerobic conditions according to the RNA-seq data. The induction profile of *cydA* in anaerobic conditions with

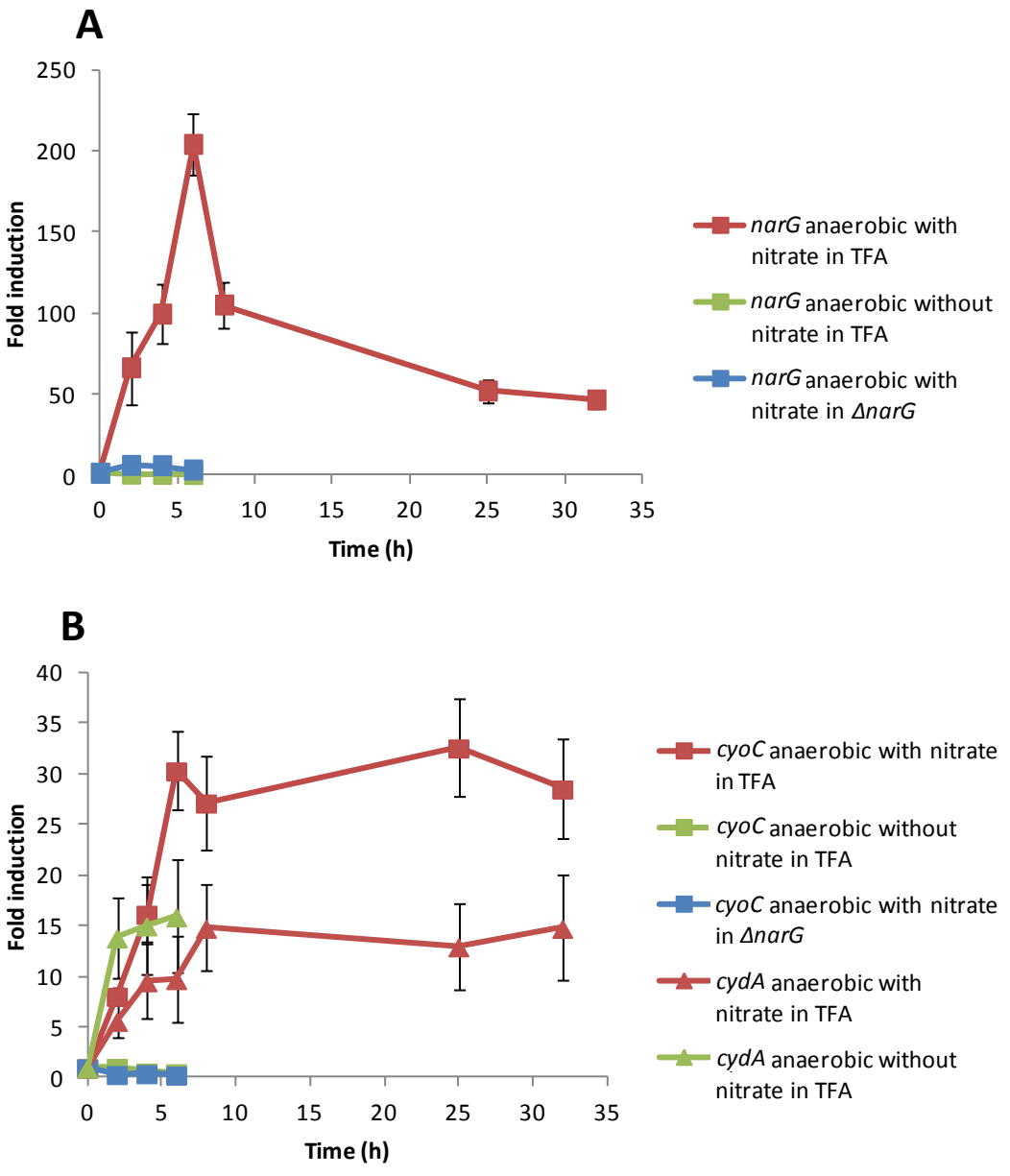
nitrate in the WT strain (Figure 18B) was very similar to that of *cyoC*, although the induction level was lower, thus reaching a maximum of 15-fold induction. However, in this case, in the absence of nitrate in anaerobic conditions, *cydA* was still induced 15-fold, being the induction even faster than in the presence of nitrate, thus suggesting that the induction of this gene does not required the presence of nitrate, just that the bacterium is in anaerobic conditions.

The cytochrome *c* oxidase cbb_3 is encoded in the operon *ccoNOQPGHIS*. These genes seem to be clustered in an operon as they are very close or even overlapping with each other, but their regulation must be complex, as just the distal part of the operon, *ccoHIS*, was induced in anaerobic conditions. This was due to the higher expression levels of the proximal genes *ccoNOQPG* in aerobic conditions compared to the distal genes. The dRNA-seq data also showed that the genes of the proximal part of the operon were inhibited in aerobic conditions by NO, which agree with the strong inhibition of cbb_3 oxidases by NO reported in other bacteria[315], and in the presence of other stress factors such as slow growth on tetralin as carbon source. Figure 18C shows that *ccoH* gene was induced in anaerobic conditions with nitrate in the WT strain up to around 10-fold in 6 hours, progressively decreasing its mRNA level after that time. In anaerobic conditions with nitrate in the Δ*narG* mutant the expression of this gene was not affected, while in anaerobic conditions without nitrate in the WT strain, *ccoH* was actually repressed 10-fold. This suggests that, as *narG*, this gene may need the presence of nitrate to be induced but nitrate respiration is essential for full induction of this alternative electron acceptor.

The alternative oxidase Aox, reported as insensitive to NO[32], was induced 5-fold in anaerobic conditions and it was also induced in aerobic conditions in the presence of NO. Figure 18D shows that *aox* was highly induced up to 450-fold in 4 hours but its induction drastically decreased after 8 hours of anaerobic growth. In the absence of nitrate in anaerobic conditions the WT strain, *aox* was induced just up to 17-fold and just up to 7 fold in anaerobic conditions with nitrate in the Δ*narG* MPO253 mutant. Interestingly when NO was added in anaerobic conditions, this gene was induced 70-fold after two hours of anaerobic incubation without nitrate (0.03 arbitrary units in time 0 hours without

NO versus 2.1 arbitrary units after 2 hours with NO), which suggest that this gene is regulated by NO.

As a control, expression kinetics of each of these genes in aerobiosis was performed along the growth curve by RT-qPCR and expression changed less than 3-fold (Table 8).



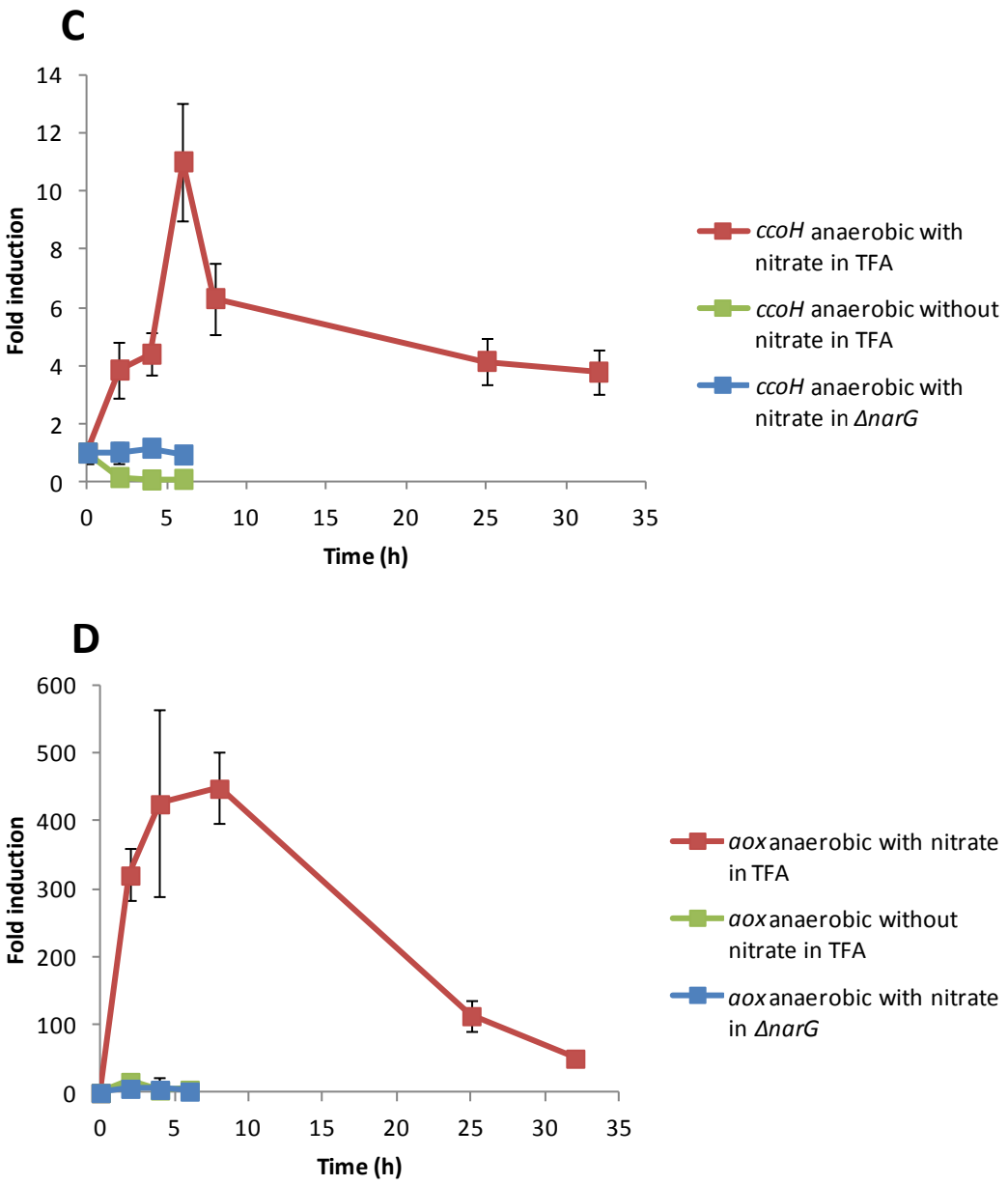


Figure 18. Induction kinetics of genes coding for electron transfer chains components. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, anaerobic conditions without nitrate in the WT strain in green and anaerobic conditions with 20 mM nitrate in the Δ*narG* mutant MPO253 in blue. Fold induction of: (A) *narG* gene; (B) *cyoC* (squares) and *cydA* (triangles) genes; (C) *ccoH* gene and (D) *aox* gene. Fold change induction of each gene over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

Gene	Maximum induction fold
narG	2.9±0.2
cyoC	1.7±0.3
cydA	1.8±0.4
ccoH	1.4±0.4
aox	1.2±0.1

Table 8. Maximum induction fold of genes coding for electron transfer chains in aerobiosis. This table shows the maximum induction fold of genes coding for electron transfer chains components in aerobiosis, obtained by RT-qPCR. The mean ± SD of 3-4 technical replicates.

2.2. Protein turnover genes

The highest induced gene of this category was found to be that putatively coding for the protease YhbU, which had practically no expression in aerobiosis and was induced 300-fold in anaerobiosis in our dRNA-seq results. The contiguous downstream gene to *yhbU*, *yhbV*, was also induced in anaerobic conditions almost 100-fold. Furthermore, the upstream gene of *yhbU*, that is annotated as uncharacterised but showed homology by protein Blast with YhbT of several *Rhodovulum* species, was also induced 190-fold in anaerobiosis. In TFA, these three proteins seem to be enconded in the operon *yhbTUV*. Out of the 20 genes coding for proteases, including ClpP and HslV systems, only Yhb proteases were induced in anaerobic conditions. While in aerobic conditions the expression of *yhbU* was very low along the growth curve, reaching a maximum induction of 1.9 fold (not shown), in anaerobic conditions with nitrate in the WT strain *yhbU* was highly induced up to 800-fold in the first 8 hours of growth, its mRNA levels progressively declining after this (Figure 19). In anaerobic conditions without nitrate in the WT and in anaerobic conditions with nitrate in the Δ*narG* mutant MPO253, this gene showed a very low induction of just 6-fold in the first 2-4 hours of anaerobiosis, thus suggesting that induction of this gene requires nitrate respiration and possibly growth under anaerobic conditions.

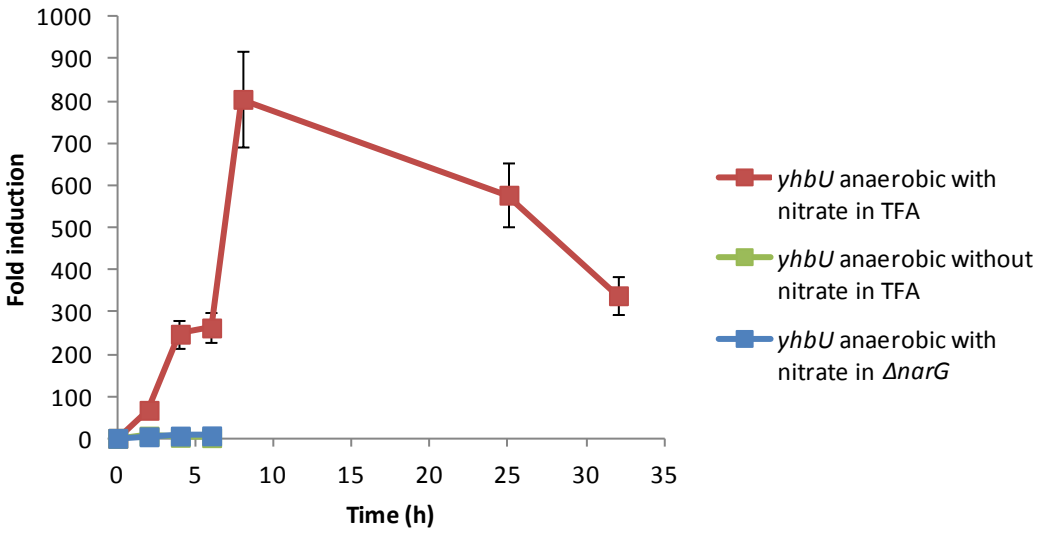


Figure 19. Induction kinetics of the *yhbU* gene, coding for a protease. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, anaerobic conditions without nitrate in the WT strain in green and anaerobic conditions with nitrate 20 mM in the Δ*narG* mutant in blue. Fold change induction of the gene over time with respect to time 0 is shown and graphic represents the mean ± SD of 3-4 technical replicates.

2.3. Stress response/detoxification

Many genes belonging to different COG categories but that are probably involved in stress response and detoxification in TFA, were induced in anaerobic conditions, according to the RNA-seq results. We observed induction in anaerobiosis of three operons coding for sulphide-quinone reductases, sulphurtransferases (some of them rhodaneses) and transport proteins, probably involved in sulphur detoxification, which is consistent with the fact that the toxic reduced form of sulphur, H_2S, is more abundant under anaerobic conditions. This suggests that there may be a mechanism in TFA that uses the absence of oxygen as a cue to activate H_2S detoxification genes. Among the detoxification genes, those comprising the operon SGRAN_3840-3842, that encodes for a sulphide-quinone reductase, a sulphurtransferase and a channel protein, were also induced by NO in aerobic conditions 3.6-, 5.4- and 5-fold, respectively.

It is also remarkable the induction in anaerobiosis of the *ectABCD* operon, which probably codes for proteins that synthesise the osmoprotectant ectoine, involved in osmotic stress response[316]. Moreover, some genes coding for universal stress proteins (SGRAN_0888, SGRAN_3353) and for an extracytoplasmic function sigma factor (SGRAN_1161) probably involved in a general stress response, were induced in anaerobic conditions but not in aerobic conditions in the presence of NO. Unexpectedly, among the stress genes induced in anaerobic conditions we found peroxidases, peroxiredoxines and a thioredoxine, which normally respond to oxidative stress.

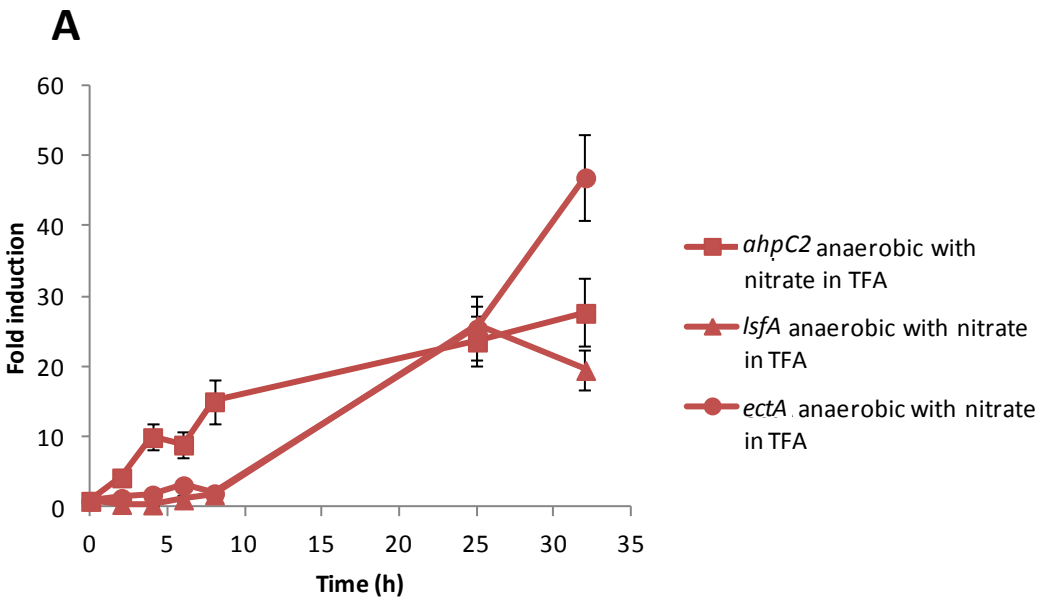
Other operons, probably coding for stress response/detoxification proteins, were induced both by anaerobiosis or the presence NO in aerobiosis, being the induction levels by NO even higher. Among them we found the arsenic resistance operon *arsR3H2C3B2*[317] and the extended nitric oxide reductase gene *norB*, which codes for a qNOR protein with the two subunits of the NO reductase fused in one[67]. The most induced operon by anaerobiosis and NO (100 to 200-fold) was SGRAN_3394-3396, which apparently codes for proteins involved in protein traffic though the membrane, though their function could not be well defined by sequence homology. It was interesting that the divergent gene *nsrR*, putatively coding for a repressor that senses NO thanks to a [2Fe-2S] cluster[134], was also induced to almost the same level by anaerobiosis (6.8-fold) and by NO in aerobiosis (4.5-fold). In a similar way, *ytfE*, that codes for a protein involved in Fe-S clusters repair[103], was highly induced both by anaerobiosis and by the presence of NO in aerobic conditions. In the same conditions there was also induction, though to lower levels, of the operon SGRAN_1371-136, consisting of a transcriptional regulator, *sufBCDS* genes, which code for the components of the Fe-S cluster biogenesis apparatus[318], and two genes coding for Fe-S assembly proteins.

The induction level over time was studied for five stress response genes, *ahpC2*[319], *lsfA*[320], *ectA*[316], *norB*[67] and *ytfE*[103], from which the last two were also aerobically induced by NO. Figure 20A shows that *ahpC2*, *lsfA* and *ectA* were all induced in anaerobic conditions with nitrate in the WT strain, though their induction was slower and progressive, reaching their maximum level at the end of the growth curve, with the exception of *ahpC2*, which seemed to reach it at

around 25 hours. This induction kinetics was slower than in previous cases, probably because they need for their induction the accumulation of the anaerobic respiration products that generate this stress situation. As for *ytfE* (Figure 20B), this gene also showed a slow induction pattern, likely because in anaerobiosis Fe-S clusters lose their Fe and need to be repaired or synthesised *de novo*, which would explain the progressive induction of the required genes.

In the case of *norB* (Figure 20C), its induction in anaerobic conditions with nitrate in the WT was very fast reaching its maximum, a 60-fold induction, at the first 2 hours, decreasing then to 40-fold and maintaining its mRNA levels stable the rest of the culture. This suggests that *norB* induction is responding to a different condition than the previously mentioned stress response genes. In anaerobic conditions without nitrate in the WT and in anaerobic conditions with nitrate in the Δ*narG* mutant MPO253 the induction was very low (4 to 8-fold), suggesting that nitrate respiration is required for the induction of this gene.

On the other hand, a two-hours incubation of TFA in anaerobic conditions in the presence of NO highly induced the expression of *norB* (24-fold) and *ytfE* (327-fold), although their different induction kinetics suggest that *norB* responds directly to NO while *ytfE* responds to the nitrosative damage caused by NO accumulation.



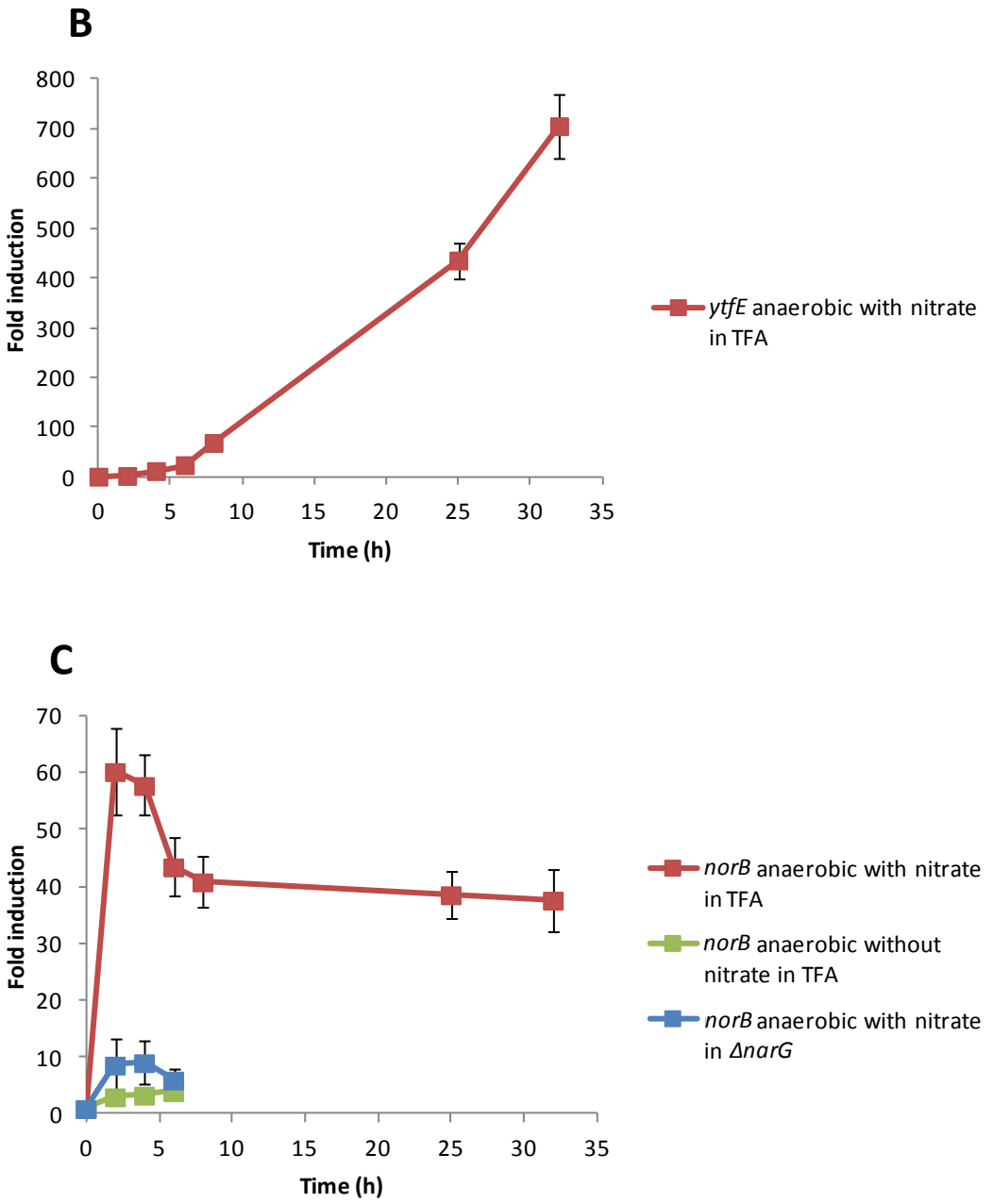


Figure 20. Induction kinetics of genes involved in stress response and detoxification. Anaerobic conditions with 20 mM in the WT strain are represented in red, anaerobic conditions without nitrate in the WT strain in green and anaerobic conditions with nitrate 20 mM in the Δ*narG* MPO253 mutant in blue. (A) Induction of *ahpC2* (squares), *lsfA* (triangles) and (circles) genes. (B) Induction of *ytfE*. (C) Induction of *norB*. As a control, expression kinetics of each gene in aerobiosis was performed along the growth curve and expression changed less than 4.5-fold. Fold change induction of each gene over time with respect to time 0 is shown and graphics represent the mean ± SD of 3–4 technical replicates.

Results

2.4. Replication, cell cycle and cell division genes

The genes coding for the well-known cell cycle regulators in α-Proteobacteria *ccrM* and *ctrA*[321] were found to be repressed 7 and 3.5-fold respectively in anaerobic conditions in TFA in our dRNA-seq results. Moreover, a gene coding for a chromosome partitioning protein (SGRAN_3445) and the genes *ftsN*, *ftsB* y *ftsW* involved in cell division[322] were also repressed 3 to 6-fold, though other *fts* genes were not differentially regulated. However, in contrast to the gene expression machinery genes (for example SGRAN_2978), the expression levels of *ctrA* were not reduced and those of *ccrM* were just slightly reduced in slow growth conditions in aerobiosis, suggesting that this repression in anaerobiosis is not a consequence of slow growth.

RT-qPCR analyses (Figure 21A) showed that the induction kinetics of *ctrA* and *ccrM* in aerobic conditions in the WT is dependent of the growth phase, increasing the expression progressively up to 15-fold in the case of *ctrA*. In anaerobiosis with nitrate in the WT strain *ctrA* was induced less than 3-fold, while in the case of *ccrM*, its expression was quickly repressed in the first two hours, then recovered after 6 hours, although not reaching the levels of aerobic conditions, to finally drop in late exponential phase.

Consistently with the slower growth rate in anaerobic conditions, some genes involved in NTPs biosynthesis, belonging to COG category F, were also repressed, such as the gene coding for the nucleoside diphosphate kinase. On the other hand, *nrdZ*, which codes for a O_2-independent ribonucleotide reductase essential for DNA synthesis in anaerobic conditions[323] was progressively induced in anaerobic conditions in the WT up to 356-fold (Figure 21B). In anaerobic conditions without nitrate in the wild type strain, however, this gene was only induced 6-times, while in the Δ*narG* mutant in anaerobic conditions with nitrate *nrdZ* expression was initially induced but it subsequently decayed as shown in Figure 21B. This suggests that *nrdZ* induction requires nitrate, but that maximal expression requires respiration.

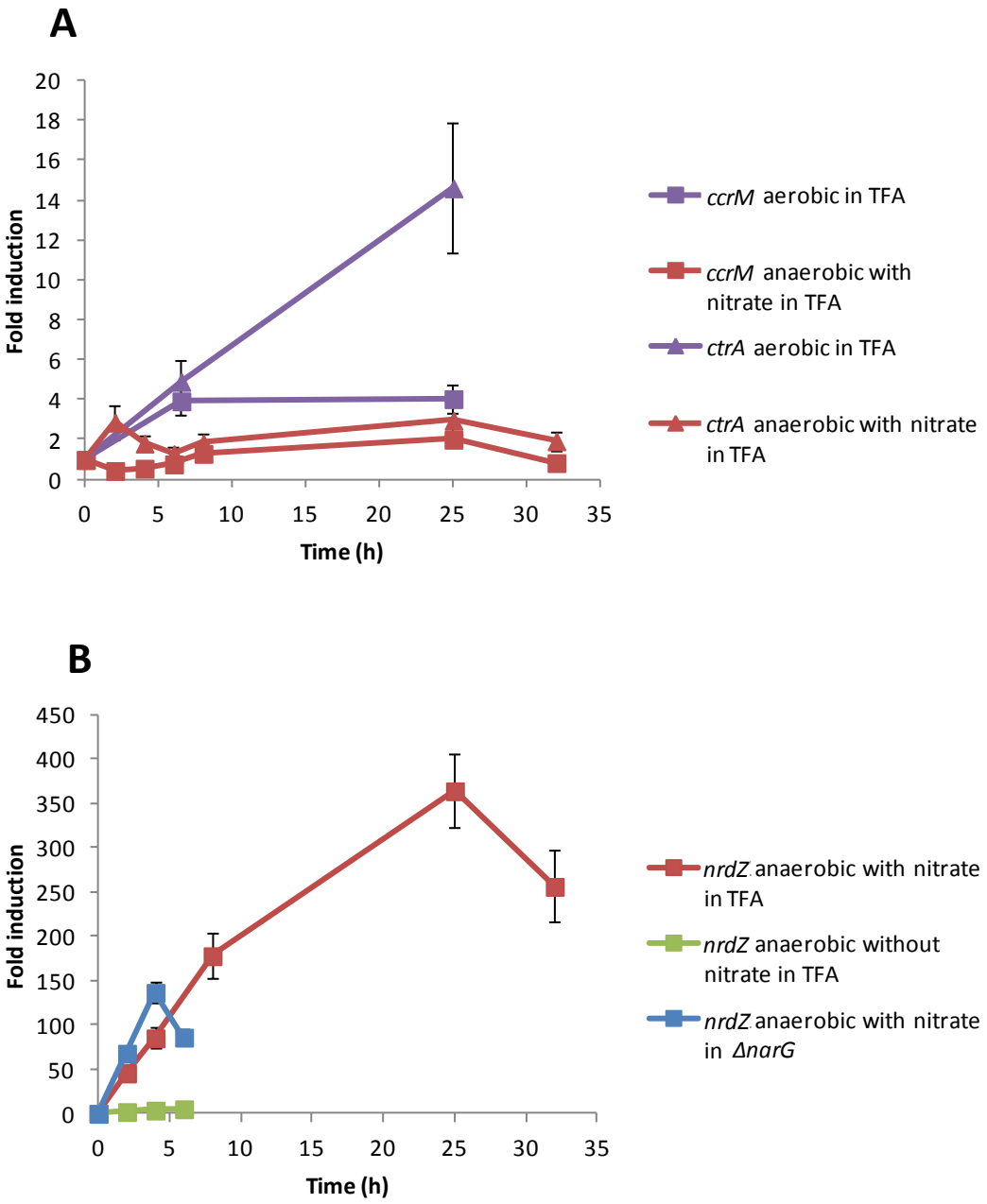


Figure 21. Induction kinetics of genes involved in replication, cell cycle control and cell division. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, aerobic conditions in the WT strain in purple, anaerobic conditions without nitrate in the WT strain in green and anaerobic conditions with 20 mM nitrate in the Δ*narG* mutant MPO253 in blue. (A) Fold induction of *ccrM* (squares) and *ctrA* (triangles) genes. (b) Fold induction of *nrdZ* gene. As a control, expression kinetics of *nrdZ* in aerobiosis was performed along the growth curve and expression changed less than 2.5-fold. Fold change induction of each gene over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

2.5. SOS DNA repair system

We found particularly interesting the induction in anaerobic conditions in TFA of many genes normally involved in SOS response to DNA damage in other bacteria. One of the genes induced in anaerobic conditions was *recA*, whose product controls LexA activity by proteolysis in response to DNA damage[178,179], the two operons that code for an error-prone DNA polymerase able to perform DNA translesion synthesis[324] *imuABdnaE2* and *imuA2B2dnaE2.1*, and genes of unknown function but that code for proteins with a SRAP domain, which acts as a DNA-associated autoproteolytic switch that recruit several repair enzymes in order to fix DNA damage[325].

We have analysed the induction kinetics of the genes *recA* and *imuA* (Figure 22), and we have observed that both genes were induced in anaerobic conditions with nitrate in the WT strain, but this induction was slow. This suggests that the induction of these genes is responding to damage in DNA, and they need that this damage is accumulated in order to induce the expression of these genes.

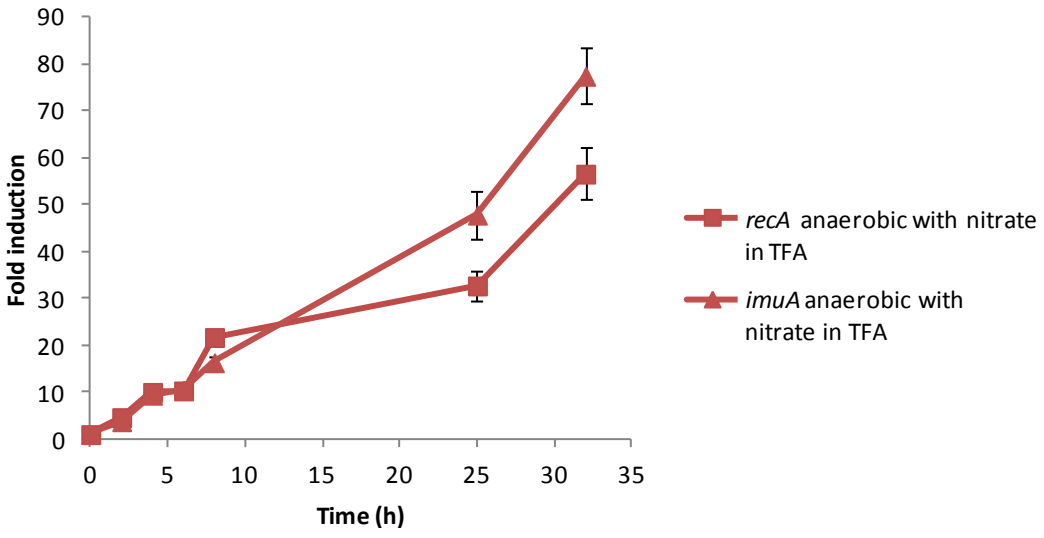


Figure 22. Induction kinetics of genes of the SOS DNA repair system. Induction of *recA* (squares) and *imuA* (triangles) in anaerobic conditions with 20 mM nitrate in the WT strain. As a control, expression kinetics of each gene in aerobiosis was performed along the growth curve and expression changed less than 3-fold. Fold change induction of each gene over time with respect to time 0 is shown and graphic represents the mean ± SD of 3-4 technical replicates.

2.6. Bacterial appendages, motility and adherence

In TFA we have found 39 putative contiguous flagellar genes (SGRAN_4088-4126), all of them repressed in anaerobic conditions in our dRNA-seq (Table 9). Furthermore, 6 out of the 8 genes putatively involved in chemotaxis annotated in TFA (*cheAWYBR* and SGRAN_3155) were also repressed in anaerobic conditions at least 5-fold (Table 9). This repression of flagellar genes in anaerobic conditions suggests that motility could be reduced in anaerobiosis (see Section 4). Induction kinetics of the flagellins *fliC* and *fliC2* analysed by RT-qPCR showed that, while they were induced in aerobic conditions in a growth phase-dependent fashion, there was no induction of these genes in anaerobic conditions along the growth curve (Figure 23).

In addition to flagellar genes, TFA also has genes coding for type IVb pili, and all the 13 genes involved in pili secretion and Flp pilus assembly (*cpa*, *tad*, *pilZ*) were also repressed in anaerobic conditions (Table 9). Figure 23 shows that the induction kinetics of the *cpaA* gene was the same as that of the flagellin genes, thus suggesting that pili genes are co-regulated with flagellar genes.

SGRAN	Gene name or product description	Fold repression
4088 and 4089	*fliC* and *fliC2* flagellins	18.8 and 22.6
4090-4104	*fliEFGHI*-SGRAN_4095-*fliKLMNOPQRflhB* operon	4-17.4
4105-4106	*fliDS* operon	7 and 16.1
4120-4107	*flgBCDEFGHIJ'KLmotABfleQ* operon	5.1-17.4
4121-4126	*flgAM*-SGRAN_4123-*flg'JflhAfliA* operon	4.1-14.4
2956–2952	*cheAWYBR* operon	5.4-11.3
3155	Methyl-accepting chemotaxis protein	9.4
1907–1901	*cpaABCDEtadBC* operon	4-12.6
3632	Flp/Fap pilin component	8.7
0765–0767	*tadGE1E2* operon	3.4-5.1
3043	*pilZ*	8.7
3775	*cpaF*	5

Table 9. Fold repression of flagellar, pili and chemotaxis genes in anaerobiosis in TFA. Table shows the SGRAN number, gene name or protein product description and fold repression in anaerobic conditions obtained by dRNA-seq of TFA flagellar, pili and chemotaxis genes.

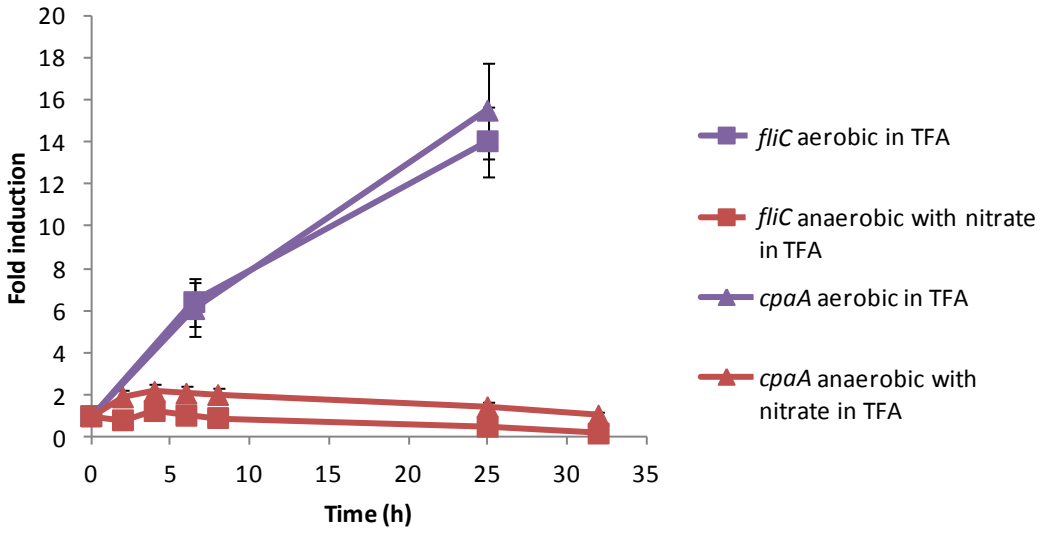


Figure 23. Regulation kinetics of flagellar and pili genes in TFA. Regulation of *fliC* (squares) and *cpaA* (triangles) genes. Aerobic conditions in the WT strain are represented in purple and anaerobic conditions with 20 mM nitrate in the WT strain in red. Fold change induction of each gene over time with respect to time 0 is shown and graphic represents the mean ± SD of 3-4 technical replicates.

2.7. Gene expression regulators

Among the genes involved in transcription regulation (COG category K) and signal transduction (T) induced in anaerobic conditions, expression of global regulators putatively responsible for the regulation of genes in anaerobic conditions was of particular interest. Expression of the genes coding for two putative anaerobic regulators, FnrN and FixK, of which we will talk with more detail in section 3, was found to be induced in anaerobic conditions, 5 and 13-fold, respectively, in the dRNA-seq analysis.

Induction kinetics analyses showed that in anaerobic conditions with nitrate both genes were induced in a progressive way, being the induction of *fixK* higher than that of *fnrN*, while in aerobic conditions both genes kept the same levels. Furthermore, in anaerobic conditions without nitrate in the WT strain *fixK* gene was not induced, thus suggesting that its induction needs the presence or respiration of nitrate (Figure 24).

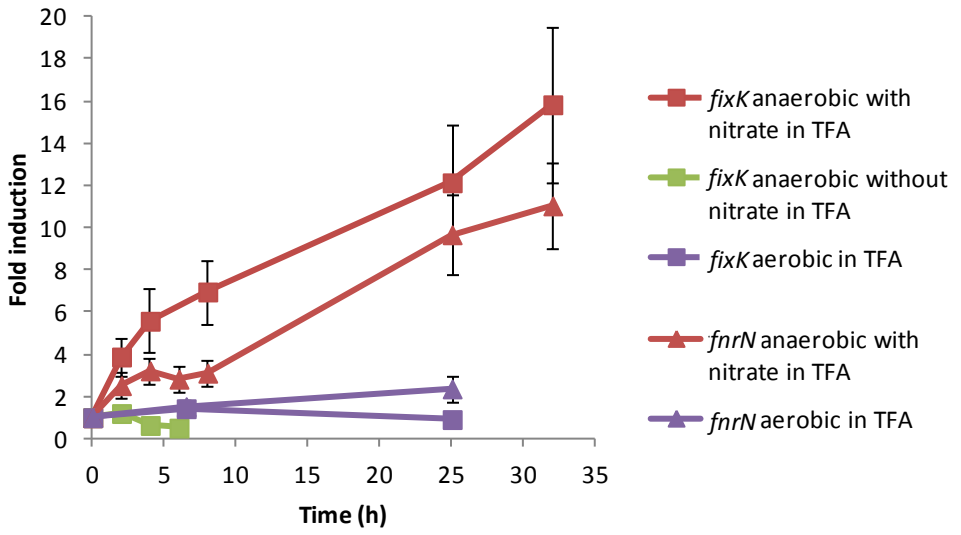


Figure 24. Regulation kinetics of gene expression regulators in TFA. Regulation of *fixK* (squares) and *fnrN* (triangles) genes. Aerobic conditions in the WT strain are represented in purple and anaerobic conditions with 20 mM nitrate in the WT strain in red and anaerobic conditions without nitrate in the WT in green. Fold change induction of each gene over time with respect to time 0 is shown and graphic represents the mean ± SD of 3-4 technical replicates.

Furthermore, 3 pairs of genes coding for putative anaerobic regulators, annotated as *fixLJ*, of which we will also talk further in section 3, were found in TFA. However, only the second operon, *fixL2J2*, was induced in anaerobic conditions, just 3.5 and 3-fold, respectively.

3. Anaerobic gene expression regulation

3.1. FixLJ system regulatory proteins

As we have seen, FixLJ is a two component system formed by the sensor hemeprotein FixL and its response regulator FixJ, that are able to detect the decrease in oxygen concentrations[78,79] and induce the expression of $fixK_2$ gene, whose protein product, in turn, induces the expression of genes in order to adapt to these conditions[80].

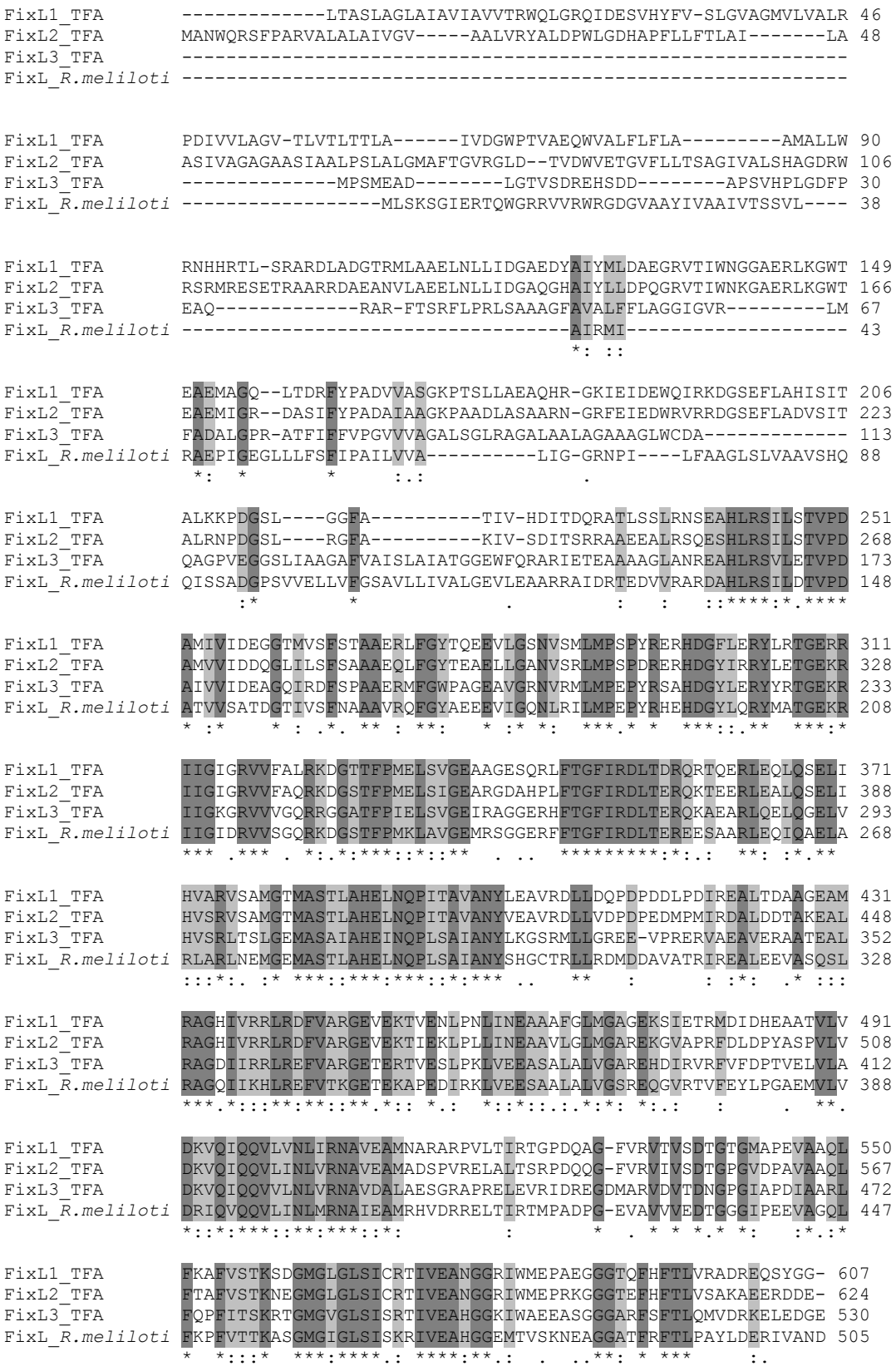
3.1.1. Sequence analysis of possible FixLJ regulatory proteins in TFA

In the first place, as we have mentioned previously, three possible pairs of anaerobic regulatory proteins annotated as FixLJ were found in TFA genome

that could be regulating the expression of genes in response to anaerobiosis. Protein alignments of FixL and FixJ proteins of TFA with the well characterised FixLJ proteins *S. meliloti*[326] were performed (Figure 25). Identities ranging between 34.5-48-3% were obtained when aligning each of the three FixL and FixJ proteins of TFA separately with those of *S. meliloti*. When the three FixL proteins of TFA were aligned together with the FixL of *S. meliloti* (Figure 25A) and the same was done with FixJ (Figure 25B), the identities were 22.5% and 32.7% respectively. The alignments showed higher identity in residues that may have functional relevance in accordance to crystallographic analyses[327,328].

Moreover, protein domains analyses using the database pfam showed that the three FixLJ proteins of TFA had the same structural domains as those of *S. meliloti*. The three putative FixL proteins of TFA showed PAS domains, two in FixL1 and FixL2 and just one in FixL3, involved in signal detection, one His kinase A domain involved in stimulus detection in two-component systems, and a GHKL domain present in multiple proteins that bind ATP. FixL protein of *S. meliloti* showed the same domains with one unique PAS domain, as in TFA FixL3. The three putative FixJ proteins of TFA showed a response regulator receptor domain and a LuxR type DNA binding domain with a helix-turn-helix characteristic structure, the same domains present in the FixJ of *S. meliloti*.

A



B

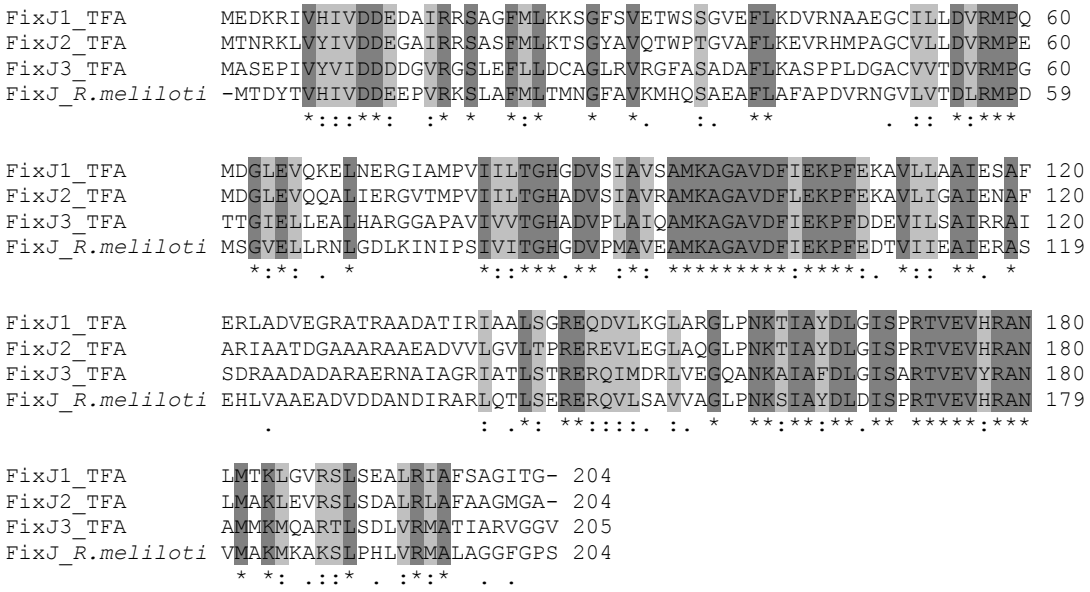


Figure 25. Protein alignments of TFA FixL and FixJ proteins with those of *S. meliloti*. The three FixL and the three FixJ proteins of TFA were aligned with the FixL and the FixJ proteins of *S. meliloti* respectively, using the Clustal Omega program. Identical residues are highlighted in dark grey and marked with an asterisk while similar residues are highlighted in light grey and marked with colon.

3.1.2. Anaerobic growth of mutants in two putative *fixLJ* system anaerobic regulators

Mutants in two of the three *fixLJ* putative genes of TFA were previously constructed in our lab[290]. In order to determine whether these two FixLJ pairs of proteins were involved in anaerobic regulation, some of these mutants were tested: a double mutant in FixJ1 and FixJ2 (MPO804), a double mutant in FixL2J2 (MPO808), a double mutant in FixL1J1 (MPO811) and a quadruple mutant in both FixL1J1 and FixL2J2 (MPO812). Their ability to grow anaerobically using nitrate as final electron acceptor was tested in rich MML medium. Results did not show significant growth differences between the mutants and the wild type control grown in the same conditions (not shown). These results suggest that these putative FixLJ proteins are not essential for anaerobic growth.

3.2. Fnr regulatory proteins

Fnr proteins are transcriptional factors that are able to sense low oxygen concentrations, undergoing a structural change that allows dimerising and becoming active. Once active, these proteins are able to bind specific sequences in the promoter regions of some genes, the FNR boxes, and regulate the expression of these genes, in order to help the cell adapt to anaerobic conditions[14].

3.2.1. Sequence analysis of possible Fnr regulatory proteins in TFA

As mentioned in section 2.7, in addition to FixLJ proteins, two putative regulatory proteins annotated as FnrN and FixK were identified. Sequence alignments of these two proteins were performed with well characterised Fnr proteins of other organisms that present the highly conserved residues of Fnr proteins: 4 cysteines in the ligand binding region and a glutamic acid, serine and arginine in the DNA binding region[88]. We observed that both TFA proteins presented these conserved residues, being the 3rd cysteine displaced 2 amino acids downstream with respect to that of *E. coli* Fnr, as it happens with FnrN-type Fnr proteins[86]. Moreover, the second helix of the helix-turn-helix motif of the DNA binding region that is highly conserved in Fnr proteins was also conserved in FnrN and FixK of TFA[88]. It is important to remark that the protein annotated as FixK in TFA showed characteristics more similar to Fnr proteins than to real FixK proteins, which do not show these conserved cysteine residues of the ligand binding site as they are not able to detect oxygen by themselves[14] (Figure 26). These data suggest that FnrN and FixK of TFA could be actual Fnr proteins.

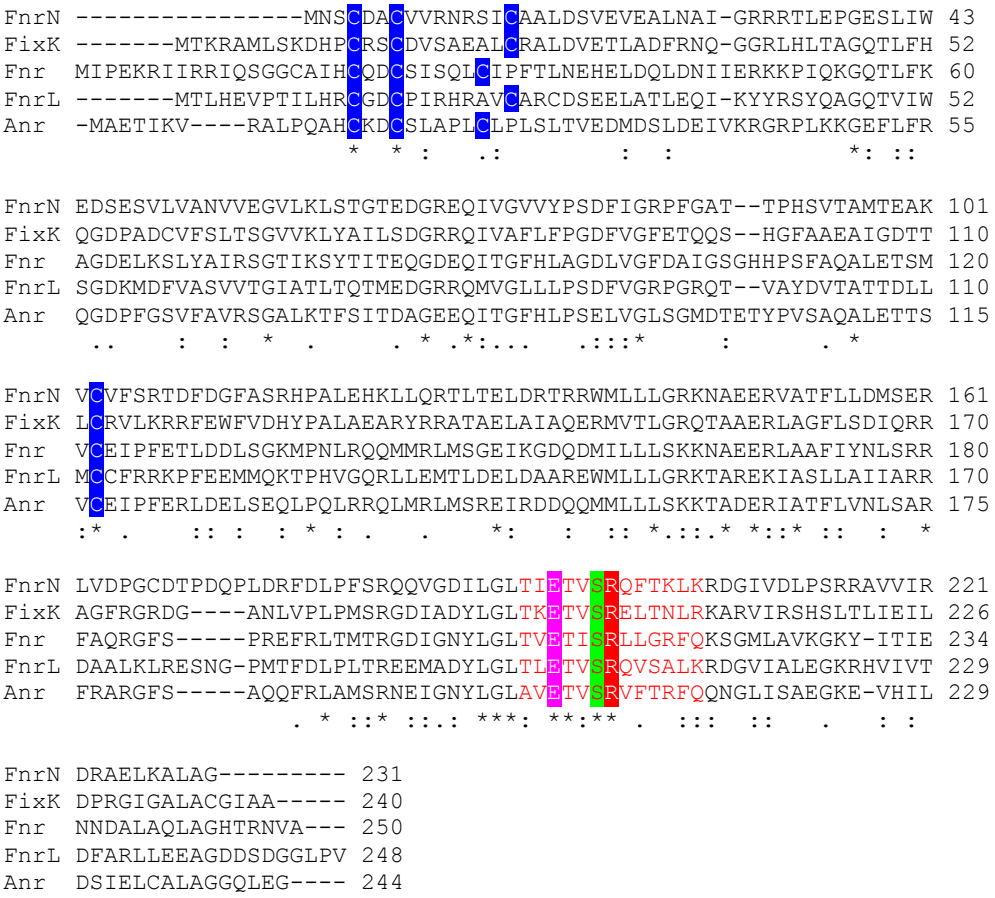


Figure 26. Alignment of TFA Fnr proteins with other conserved Fnr. TFA FnrN and FixK proteins are showed aligned with Fnr of *Escherichia coli*[329], FnrL of *R. sphaeroides*[330] and Anr of *P. aeruginosa*[331]. The 4 most conserved cysteines of the ligand binding region are highlighted in blue while the conserved glutamic acid, serine and arginine of the second helix, marked with red letters, of the helix-turn-helix domain of the DNA binding region are highlighted in pink, green and red respectively.

3.2.2. Functional complementation of *E. coli* Δ*fnr* mutant with TFA *fnrN* and *fixK*

In order to determine whether FnrN and FixK of TFA were Fnr proteins involved in anaerobic regulation, the corresponding genes were cloned separately in the expression vector pIZ1016, thus generating pMPO704 and pMPO705, respectively. These two vectors were introduced in the JRG6348 strain of *E. coli*, an *fnr* mutant strain unable to respire anaerobically, in order to test their ability to functionally complement this mutation in *E. coli*. As controls, the plasmid pGS2350 expressing the *E. coli fnr* gene was also introduced in JRG6340, and the *E. coli* Fnr^+ strain M182 carrying the empty vector was also

tested. A mineral medium with nitrate and glycerol, a non-fermentable carbon source, was used, as *E. coli* is able to ferment anaerobically. Growth was tested with and without IPTG in the medium, as in pMPO704 and pMPO705 *fnrN* and *fixK* are transcribed from a heterologous promoter induced by IPTG (Figure 27). As expected, JRG6348 mutant was unable to grow in these conditions while the *E. coli fnr* gene was able to complement the mutation and restore its growth. Results show that both TFA genes, *fnrN* and *fixK*, were able to complement the *E. coli* mutation in *fnr* when their expression was induced by IPTG and even without induction in the case of FnrN, although less efficiently. We also observed that *fnrN* complemented the mutation better than *fixK*, as *fnrN* induced by IPTG recovered growth after a lag phase of around 9 hours, as it also happened with the complementation with *E. coli fnr*, while *fixK* induced by IPTG did not recover growth until around 32 hours of incubation. In addition, when there was no induction with IPTG, the mutant complemented with *fnrN* was able to grow after around 30 hours of incubation while the complementation with *fixK* was unable to recover growth.

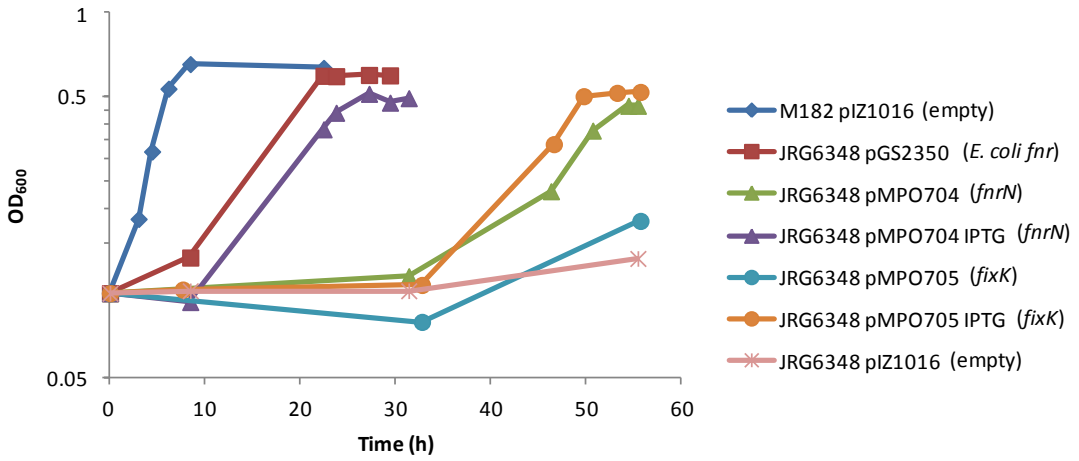
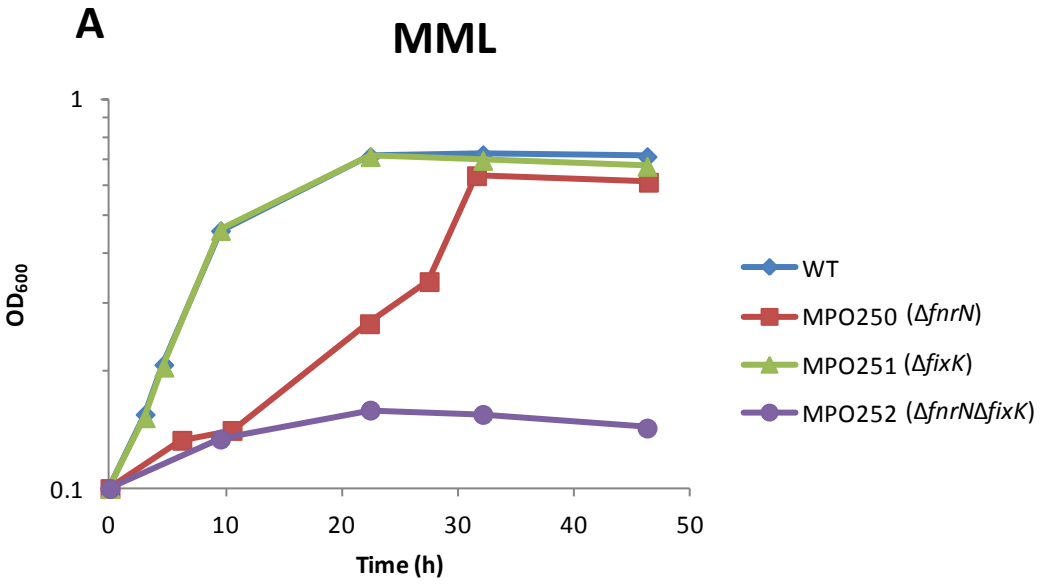


Figure 27. Functional complementation of *E. coli* with TFA *fnr* genes. Anaerobic growth, represented as OD_{600} over time, is shown for M182 with the empty vector pIZ1016 (diamonds), JRG6348 complemented with pGS2350 (squares), JRG6348 complemented with pMPO704 (triangles) with (purple) and without (green) IPTG, JRG6348 complemented with pMPO705 (circles) with (orange) and without (pink) IPTG and JRG6348 with the empty vector pIZ1016. All curves were repeated at least twice and one of the biological replicates of each curve is represented here.

3.2.3. Phenotype of TFA mutants in *fnrN* and *fixK* under anaerobiosis

Once we learnt that TFA FnrN and FixK were able to complement an *E. coli fnr* mutant, we decided to construct TFA mutants in these genes. We constructed two single deletion mutants, Δ*fnrN* (MPO250) and Δ*fixK* (MPO251), and a double mutant lacking both genes (MPO252). We tested anaerobic growth with nitrate of these three mutants comparing them to the wild type strain both in rich MML medium and in mineral medium with β-HB (Figure 28, A and B respectively). The Δ*fixK* mutant MPO251 showed very similar growth pattern to the wild type strain in both media, but the Δ*fnrN* mutant MPO250 was able to grow only in MML and after a long lag phase. The double mutant MPO252 showed the strongest phenotype since it was completely unable to grow in any of the media. These results and the one shown above suggest that both proteins, FnrN and FIxK, are real Fnr proteins in TFA, and that FnrN is more relevant for anaerobic growth than FixK.



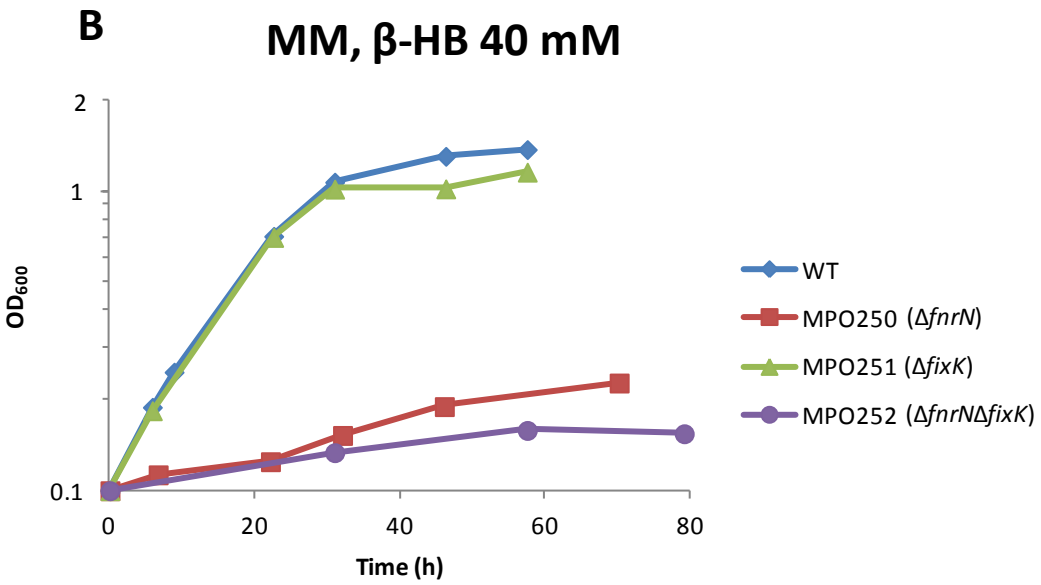


Figure 28. Anaerobic growth curves of TFA Δ*fnr* mutants. Anaerobic growth, represented as OD_{600} over time, of TFA (blue), Δ*fnrN* mutant MPO250 (red), Δ*fixK* mutant MPO251 (green) and the double Δ*fnrN*-Δ*fixK* mutant MPO252 (purple) in rich MML (A) and mineral (B) media. All curves were repeated at least three times and one of the biological replicates of each curve is represented here.

3.2.4. Functional complementation of TFA Δ*fnr* mutants with TFA *fnr*N and *fixK*

In order to see whether these TFA *Δfnr* mutants could be functionally complemented with the TFA *fnr* genes previously cloned in the vectors, we introduced pMPO704, expressing *fnrN*, and pMPO705, expressing *fixK*, in the double mutant MPO252, and the vector pMPO704 in the Δ*fnrN* mutant MPO250. No complementation assay was performed with the Δ*fixK* mutant MPO251 as it already showed a growth phenotype very similar to the wild type. Complementation assays were performed under anaerobic conditions both in rich MML and in mineral media with nitrate. As *fnrN* and *fixK* were expressed under a promoter inducible by IPTG in the vector, complementation assays were performed both in the presence or absence of IPTG 1 mM.

In the complementation assays performed with the double MPO252 mutant, results showed that in MML (Figure 29A) both pMPO704 (*fnrN*) and pMPO705 (*fixK*) were able to complement to some extent the mutation, being this complementation better with *fnrN* than with *fixK* and with IPTG than without it for

both genes. On the other hand, in mineral medium with β-HB (Figure 29B), only *fnrN* was able to clearly complement the mutation, and this complementation was slightly better when IPTG was added to the culture.

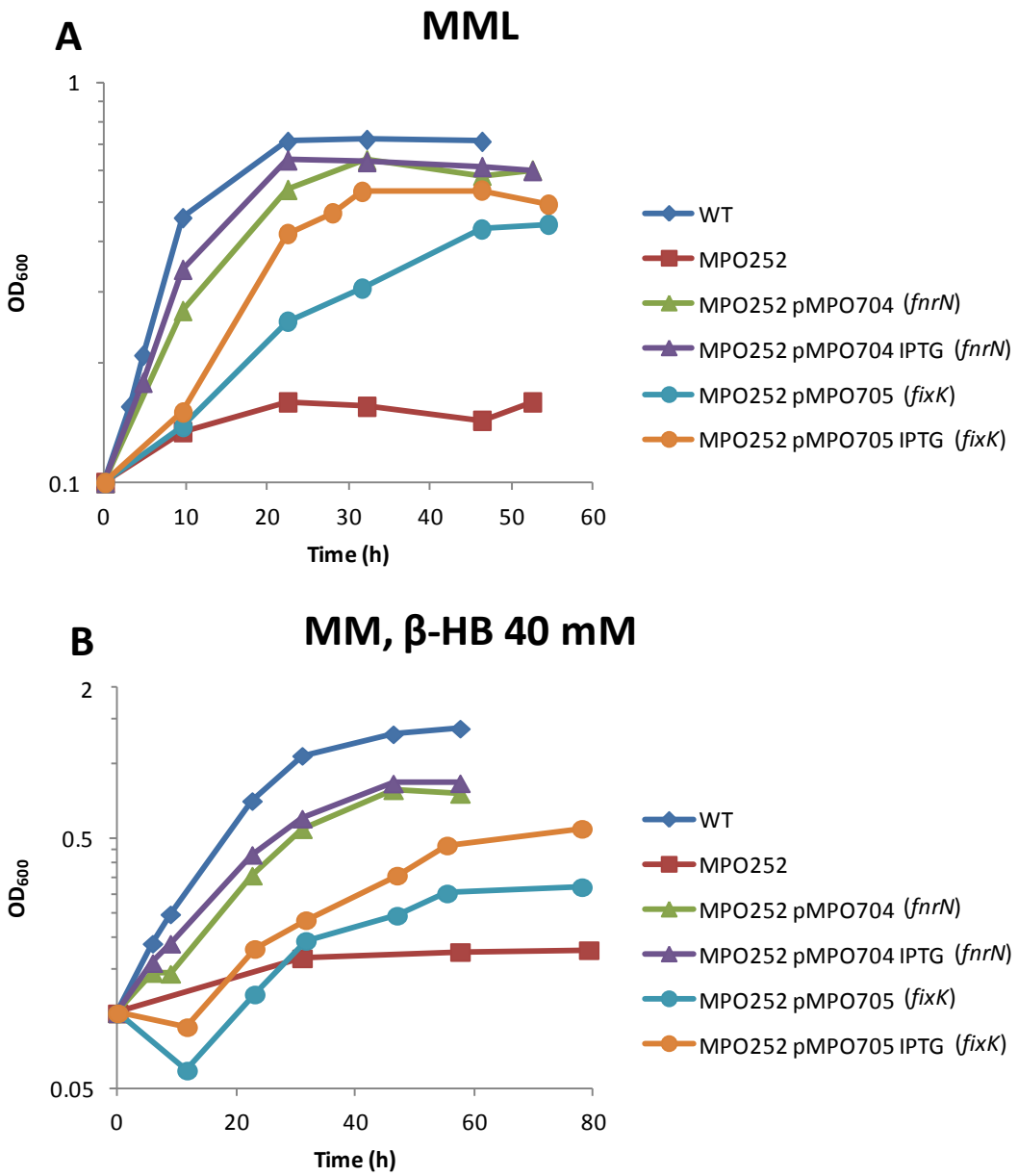


Figure 29. Functional complementation of MPO252 (Δ*fnrN*Δ*fixK*) with *fnrN* and *fixK*. Anaerobic growth, represented as OD_{600} over time, in rich medium MML (A) and mineral medium with β-HB (B) is shown for TFA (diamonds), the double mutant MPO252 (squares), MPO252 complemented with pMPO704 (triangles) with (purple) and without (green) IPTG and MPO252 complemented with pMPO705 (circles) with (orange) and without (pink) IPTG. All curves were repeated at least twice and one of the biological replicates of each curve is represented here.

As expected, regarding the complementation of the Δ*fnrN* mutant MPO250, we observed that pMPO704 (*fnrN*) was able to complement the mutation both in MML (Figure 30A) and in mineral medium with β-HB (Figure 30B), being the complementation better in MML medium, and in this case the addition of IPTG to the culture did not have an effect in the complementation efficiency.

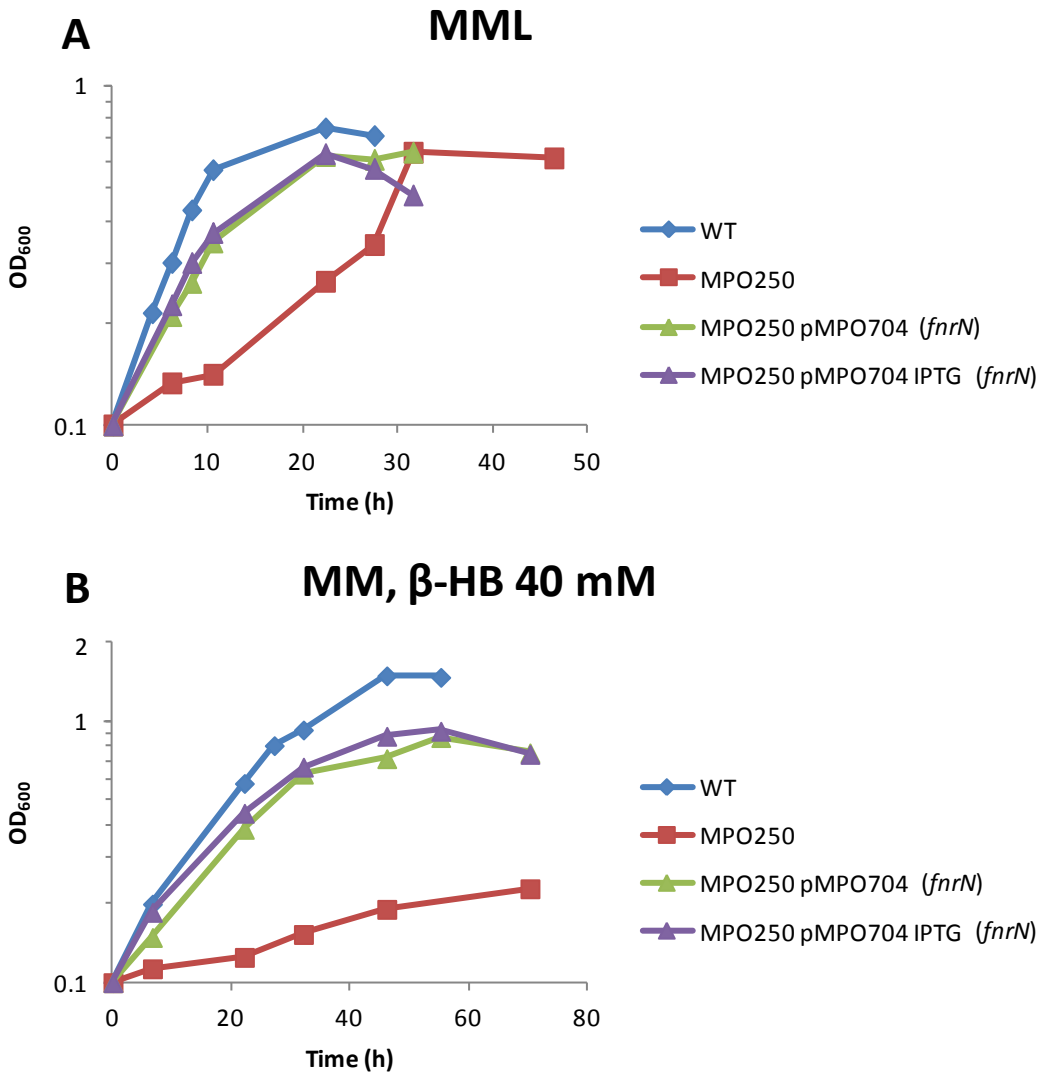


Figure 30. Functional complementation of Δ*fnrN* with *fnrN*. Anaerobic growth, represented as OD_{600} over time, in rich medium MML (A) and mineral medium with β-HB (B) is shown for TFA (diamonds), the *fnrN* MPO250 (squares) and MPO250 complemented with pMPO704 (triangles) with (purple) and without (green) IPTG. All curves were repeated at least twice and one of the biological replicates of each curve is represented here.

Altogether, these data indicate that *fnrN* gene complemented the mutation in *fnr* genes better than *fixK*, being this complementation generally better in rich medium and in the

presence of IPTG (over-expression). This view supports the results obtained in the complementation of the *E. coli fnr* mutant, which suggested that FnrN is more relevant for anaerobic growth than FixK.

3.3. Fnr regulon

3.3.1. dRNA-seq and RT-qPCR

In order to determine which genes could be regulated directly by the Fnr proteins of TFA FnrN and FixK and define its Fnr regulon, we performed a new dRNA-seq of the double Δ*fnr* mutant MPO252. As this mutant is unable to grow anaerobically, besides the genes regulated by Fnr proteins, the expression of a number of genes would be affected as a consequence of the lack of respiration and growth. For this reason, we also performed an dRNA-seq of the Δ*narG* mutant MPO253, unable to respire and grow anaerobically but not affected in regulation. Comparing these two mutants, we intended to discern which genes were regulated directly or indirectly by Fnr protein and which ones as a consequence of lack of respiration and anaerobic growth. The results of these dRNA-seq are shown in the Appendix.

Since the dRNA-seqs of the WT strains and those of the mutants were performed in different times and by different procedures, in order to be able to meaningfully compare them, we first analysed the genes that were not affected in anaerobic conditions or affected less than 2-fold. We observed that expression of most of these genes not regulated by anaerobiosis was neither affected in the Δ*fnr* nor in the Δ*narG* mutant, as expected. Altered expression in the few affected genes could be a consequence of changes on the physiology of the mutant cells as well as the difference in the conditions of the dRNA-seqs, as the WT in anaerobic conditions was analysed after 25 h of anaerobic growth while the mutants were analysed after 6 hours of anaerobic incubation. Therefore, these previous verifications validate the comparisons between the different dRNA-seqs.

We then compared expression in the WT strain to those in the mutants, analysing which of the genes affected in anaerobic conditions in the WT showed a large difference in fold-change in the mutants, in comparison with the

rest of genes of the genome. Still, there are many genes anaerobically regulated whose expression did not seem to be affected in the mutants, but a large number of them actually showed a drastic change in their expression levels.

Genes more induced in Δ*narG* than in Δ*fnr*

We found that 27 genes, which were at least 4-fold more induced in anaerobic conditions in the WT strain, lost this induction in anaerobic conditions in the double Δ*fnr* mutant MPO252 but didn't lose it or lost it to a much lesser extent in the Δ*narG* mutant MPO253 (at least 4 fold more expressed in Δ*narG* than in Δ*fnr*), being these the genes which could be most probably regulated by anaerobiosis and Fnr proteins in TFA.

We have seen that, as expected, most *nar* genes coding for the nitrate reductase belong to this group, being induced in anaerobic conditions and losing this induction in the Δ*fnr* mutant but much less in Δ*narG*. However, in our RT-qPCR analyses of *narG* (Figure 31A), we observed that although this gene was not induced in anaerobic conditions in the Δ*fnr* mutant it was just very slightly induced in the Δ*narG* mutant (a maximum of 6-fold), as compared to the induction in the WT strain, thus suggesting that induction of the *nar* operon needs nitrate respiration.

We have also seen this behaviour in the final part of the operon *cco* (*ccoHI*), which was induced in anaerobiosis, lost this induction in the Δ*fnr* mutant, but lost it to a much lesser extent in the Δ*narG* mutant. Induction kinetics of *ccoH* gene by RT-qPCR (Figure 31B) showed that in anaerobic conditions with nitrate in Δ*fnr* this gene expression was actually repressed up to 5-fold, while in Δ*narG* the expression of this gene was constant along the anaerobic incubation time. Again, it seems that the induction of this gene needs nitrate respiration. Gene *ccoS* also showed the same expression pattern in our dRNA-seq results, therefore it was ascribed to this group. However, it had very low expression, being one of the 1062 genes with the lowest base mean.

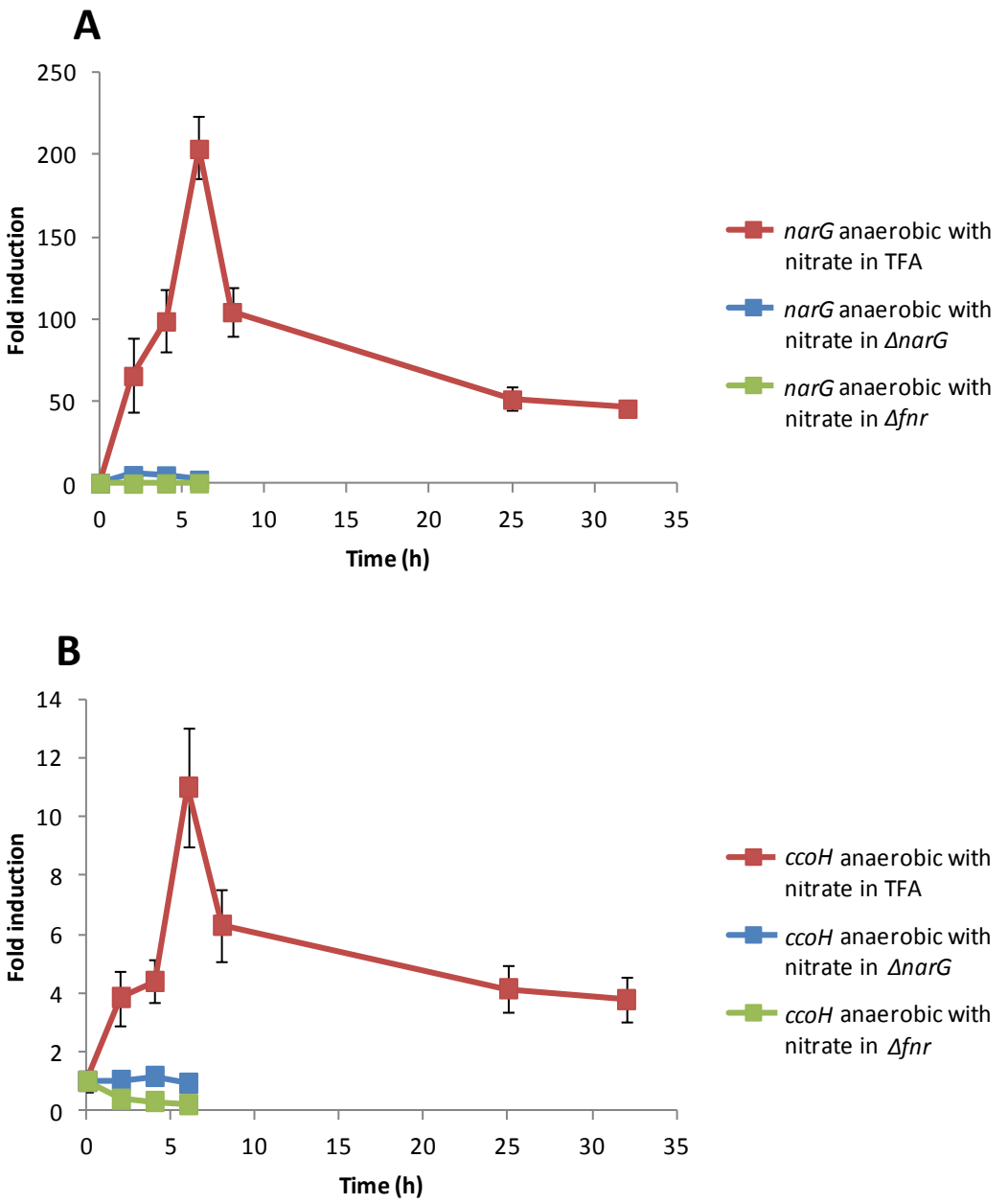


Figure 31. Induction kinetics of *narG* and *ccoH* genes in the Δ*narG* and Δ*fnr* mutants. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, anaerobic conditions with nitrate in the Δ*narG* mutant MPO253 in blue and anaerobic conditions with nitrate in the double Δ*fnr* mutant MPO252 in green. (A) Fold induction of *narG* gene. (B) Fold induction of *ccoH* gene. Fold change induction of each gene over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

The ribonucleoside reductase *nrdZ* was also included in this group, since it was 19-fold more expressed in Δ*narG* than in Δ*fnr*. In Figure 32A our RT-qPCRs shows that, in this case, this gene was substantially induced in Δ*narG*, but there

was no induction in the Δ*fnr* mutant, which confirms the results obtained in the dRNA-seq assays. These results suggest that this gene could be actually regulated by the Fnr proteins of TFA and does not need nitrate respiration, though it also seems that the presence of nitrate is necessary, since, as we saw previously, the WT could not anaerobically induce this gene in the absence of nitrate (Figure 21B).

Moreover, other genes that also belong to this category are *fixK*, several genes involved in Molibdenum cofactor biosynthesis, and, interestingly, SGRAN_3353, coding for an Universal Stress Protein.

If we take into account also genes that, despite not being induced in anaerobiosis, had a high regulation difference between Δ*narG* and Δ*fnr*, we find other genes that could be regulated by Fnr such as the cytochrome *cybB*[332], that was barely induced in anaerobiosis or in Δ*fnr*, but was highly expressed in Δ*narG*. This gene may be induced by starvation, but also need Fnr proteins for induction.

There is a subgroup of these genes that were also induced by NO in aerobiosis to the same extent or even more, as it is the case of *nnrS2* regulatory gene, whose induction decreased a lot in Δ*fnr* but not in Δ*narG*. Our RT-qPCR results (Figure 32B) show that this gene was induced very fast in anaerobic conditions with nitrate in the WT strain, 40-fold, almost reaching its maximum induction in the first two hours of growth, while in the absence of nitrate it was not induced but even repressed up to 3 fold. In Δ*narG* mutant *nnrS2* gene was slightly induced up to almost 6-fold and in Δ*fnr* mutant this gene was not induced. This suggests that induction of this gene needs that nitrate is respired.

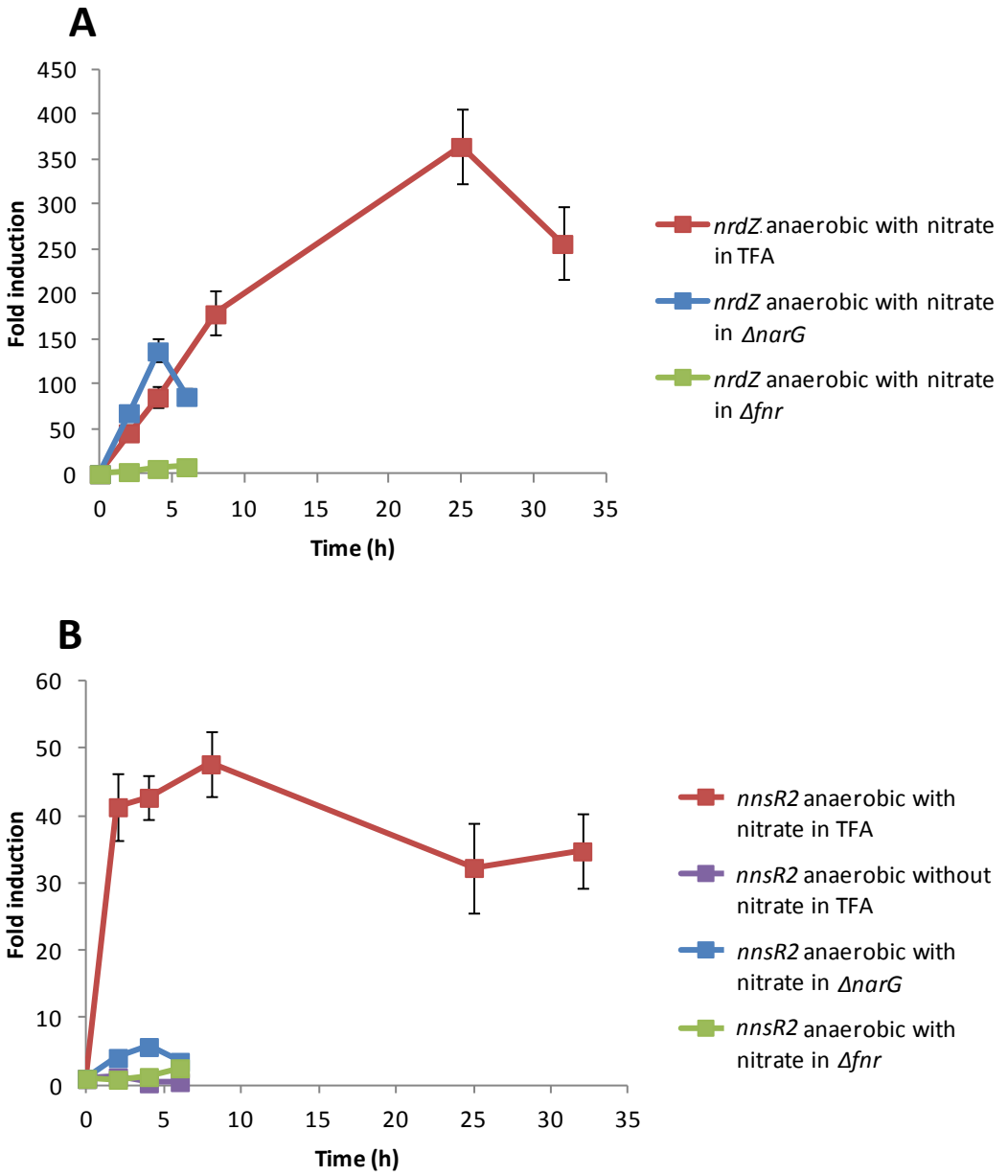


Figure 32. Induction kinetics of *nrdZ* and *nnsR2* genes in TFA without nitrate and the Δ*narG* and Δ*fnr* mutants. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, anaerobic conditions without nitrate in the WT strain in purple, anaerobic conditions with nitrate in the Δ*narG* mutant MPO253 in blue and anaerobic conditions with nitrate in the double Δ*fnr* mutant MPO252 in green. (A) Fold induction of *nrdZ* gene. (B) Fold induction of *nnsR2* gene. Fold change induction of each gene over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

Genes induced in anaerobiosis that lost their induction in Δ*fnr* and in Δ*narG*

Our dRNA-seq shows the presence of 97 genes that were induced in anaerobic conditions but that lost this induction both in the Δ*fnr* double mutant MPO252 and in the Δ*narG* mutant MPO253. Among these genes are the *cyo* operon or the genes encoding for the LsfA peroxidase[320] and the CysI2 sulphite reductase[333]. The Fnr proteins might be able to activate expression of these genes, but this possible activation would be masked by the need of anaerobic growth for induction. Alternatively, these genes might be indirectly induced by changes in environmental conditions associated to anaerobic growth on nitrate.

The operon coding for the proteases YhbU and YhbV, induced in anaerobic conditions, lost this induction in the Δ*fnr* mutant, decreasing it also a lot in Δ*narG* mutant. We have analysed by RT-qPCR the induction kinetics of *yhbU* in Δ*fnr* (Figure 33A) and we have observed that its expression was not induced in anaerobic conditions with nitrate, as it also happened in Δ*narG* mutant. This suggests that this gene needs for its induction that the bacterium is able to grow.

In our dRNA-seq the alternative oxidase *aox*, that was induced in anaerobic conditions in the WT strain, was induced almost 5-fold in Δ*fnr* with respect of the WT, but was not induced in Δ*narG*. However, in our RT-qPCR induction kinetics (Figure 33B) this gene was barely induced both in Δ*fnr* double mutant and in Δ*narG* mutant in comparison to the wild type, just a maximum of 14-fold and 7-fold respectively in contrast to the 450-fold induction of the WT strain. These results suggest that the induction of this gene requires respiration of the bacteria, but they do not give us information about whether it could be regulated by Fnr or not.

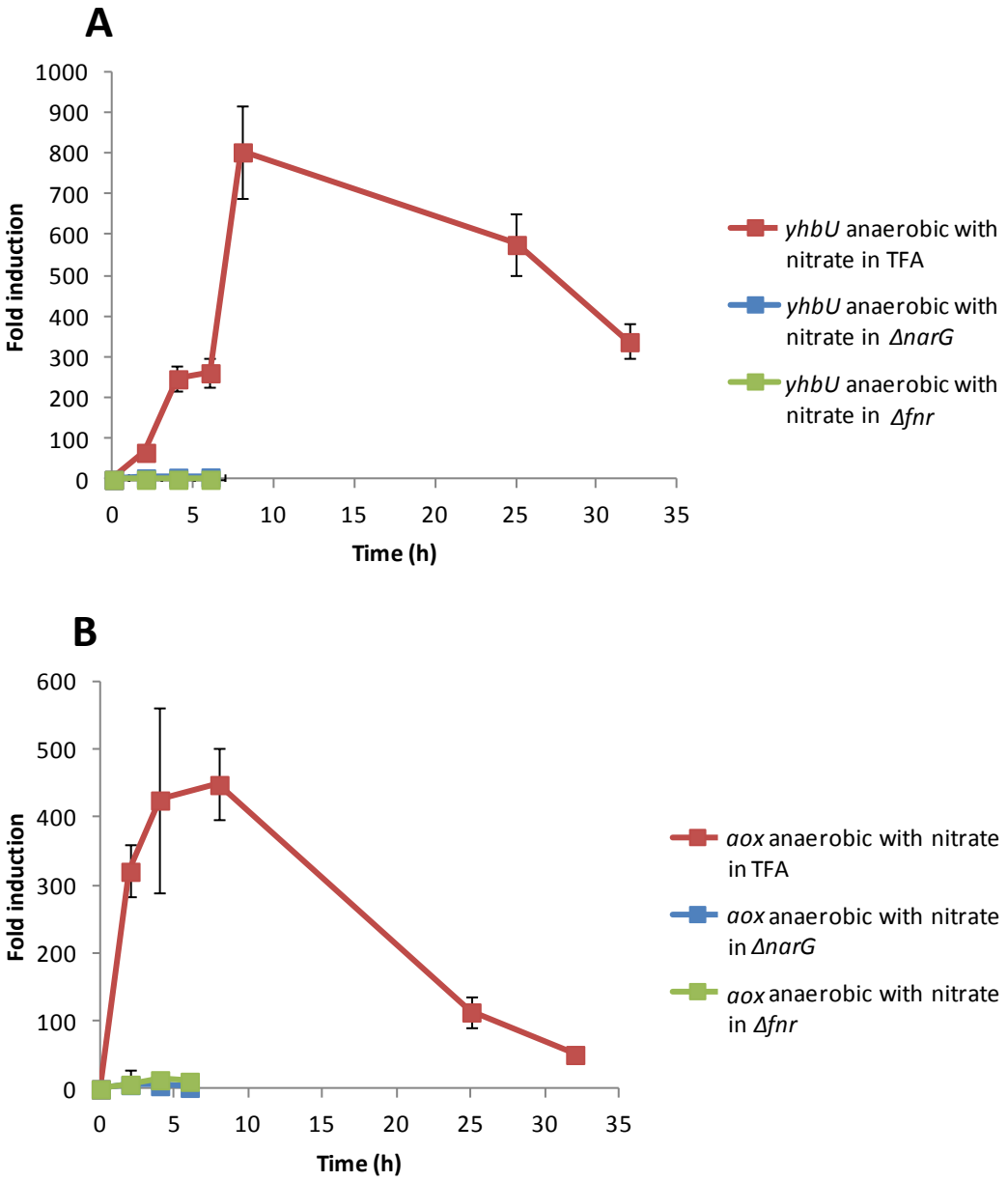


Figure 33. Induction kinetics of genes induced in anaerobiosis that lost their induction in Δ*fnr* and in Δ*narG*. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, anaerobic conditions with nitrate in the Δ*narG* mutant MPO253 in blue and anaerobic conditions with nitrate in the double Δ*fnr* mutant MPO252 in green. (A) Fold induction of *yhbU* gene. (B) Fold induction of *aox* gene. Fold change induction of each gene over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

Inside this group, there is a sub-group of 42 genes, including Phasin (SGRAN_1294), PspC domain protein (SGRAN_3152) and SGRAN_1161, induced by anaerobiosis, that lost their induction in Δ*fnr* (4 to 6-fold) but they lost it to a even higher extent in Δ*narG*. Furthermore, 11 out of these 42 genes

were also aerobically induced by NO. A possible explanation is that these genes could be induced in Δ*fnr* by NO produced by the basal activity of the nitrate reductase, that is not produced in Δ*narG* as one of the subunits of this enzyme is mutated. More examples of genes of this sub-group are operon SGRAN_1370-1368 (FeS cluster biogenesis), the preprotein translocase subunit TatC[334] (SGRAN_3394) and YtfE. Previous induction kinetics of *ytfE* showed that it was induced slowly in anaerobic conditions in the WT strain, being induced in the first 6 hours of growth, just 20-fold compared to the final 700-fold induction at longer times (Figure 20B). Therefore, this subgroup of genes is probably induced as a consequence of NO production and nitrosative damage.

Genes induced in anaerobiosis but induced further in Δ*narG* or Δ*fnr*

We also found 26 genes that, despite being induced in anaerobic conditions in the WT strain, were even more highly induced in Δ*narG* MPO253 mutant and Δ*fnr* MPO252 double mutant. SGRAN_0596, SGRAN_0597, SGRAN_0598, belonging to two divergent operons, SGRAN_0596-0593 and SGRAN_0597-0601, involved in detoxification, mainly sulphur detoxification, were induced between 7 and 10-fold more in Δ*fnr* and between 5 and 10 fold more in Δ*narG* than in the WT in anaerobic conditions. Induction kinetics by RT-qPCR shown in Figure 34A, indicate that SGRAN_0596 was induced in anaerobic conditions with nitrate in the WT strain in a slow and progressive way, reaching a maximum of 30-fold induction at 32 hours. Both in anaerobic conditions without nitrate in the WT and in anaerobic conditions with nitrate in Δ*fnr* its induction was faster, but in anaerobic conditions with nitrate in the Δ*narG* mutant this induction was tremendously fast and high, reaching an 80-fold induction in 4 hours. In the case of SGRAN_0597 (Figure 34B), it showed an induction kinetics similar to that of SGRAN_0596 in anaerobic conditions with nitrate in the WT. We can also see a fast induction in the mutants and in the WT without nitrate in anaerobiosis. This suggests that these genes could be induced by the stress caused by energy limitation or by the accumulation of NO.

Other genes belonging to this group are *trxC* thioredoxin[335], *ahpC2* peroxiredoxin (induced in anaerobiosis and even further in anaerobiosis without

nitrate) and *cyd* genes (also induced in anaerobiosis without nitrate). It seems that these genes are induced in conditions where they cannot grow because of lack of energy. However none of them were aerobically induced by carbon starvation or DETA-NO, which should inhibit aerobic respiration.

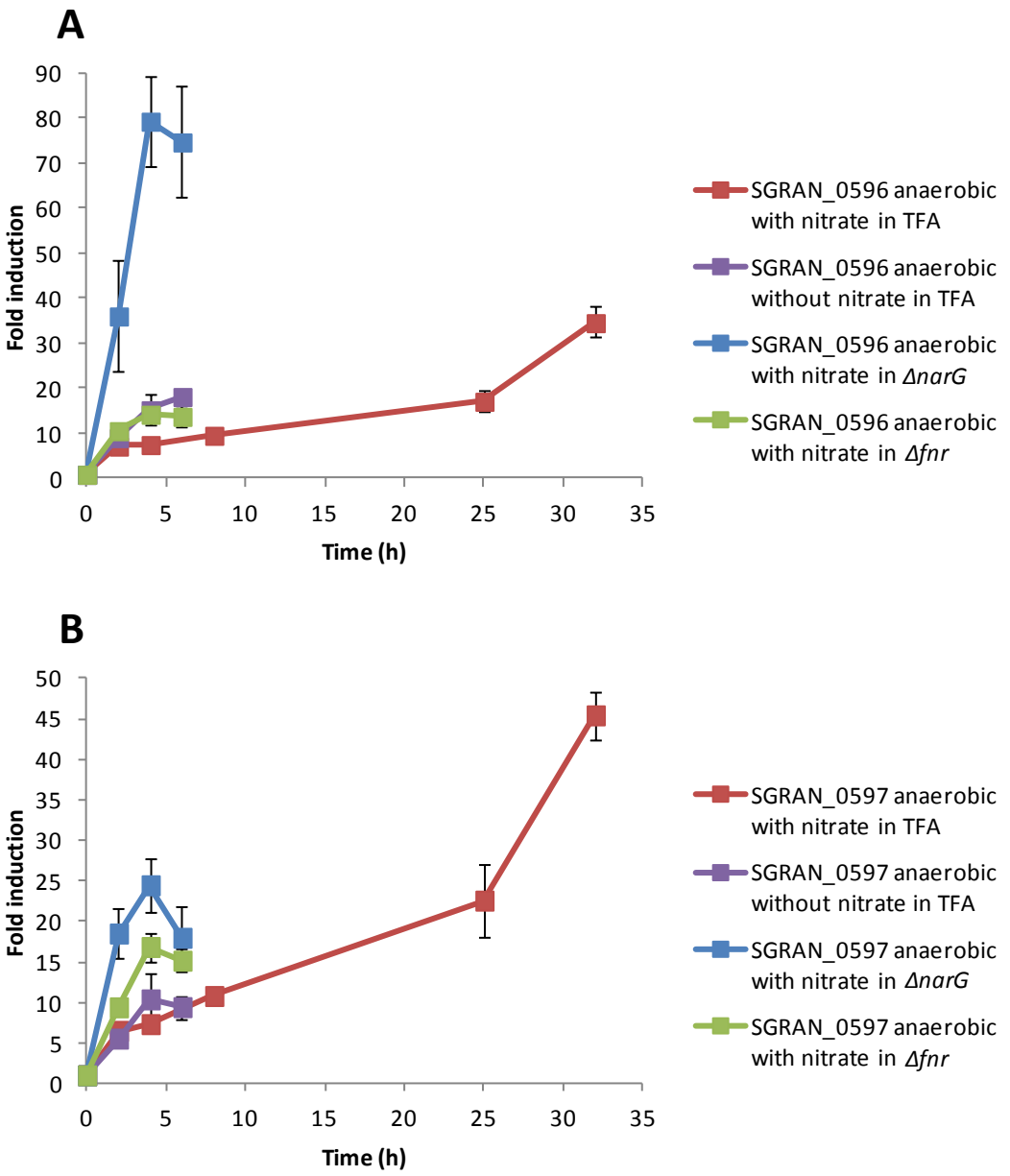


Figure 34. Induction kinetics of genes induced in anaerobiosis but induced further in Δ*narG* or Δ*fnr*. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, anaerobic conditions without nitrate in the WT strain in purple, anaerobic conditions with nitrate in the Δ*narG* mutant MPO253 in blue and anaerobic conditions with nitrate in the double Δ*fnr* mutant MPO252 in green. (A) Fold induction of SGRAN_0596. (B) Fold induction of SGRAN_0597. Fold change induction of each

gene over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

Genes repressed in anaerobiosis that lost this repression in Δ*fnr*

In the dRNA-seq analysis, 55 genes were found that, being repressed in anaerobiosis in the WT strain, lost this repression in MPO252 Δ*fnr* double mutant, which suggests that these genes could be repressed by Fnr. Among the genes that showed this behaviour there were the putative anaerobic regulator FleQ, the prepilin peptidase CpaA and the cell cycle regulator CtrA. The gene *ccrM*, also involved in cell cycle regulation, also showed a lower expression in the Δ*fnr* mutant, but its expression in Δ*narG* was slightly higher than in the WT. Flagellar genes also lost part of this repression in Δ*fnr* mutant, but practically all of them also lost it to a similar extent in Δ*narG* mutant. These results suggest that these genes are not actually repressed by Fnr, and that the lost of their repression is a consequence of not being able to grow.

Genes much more expressed in Δ*fnr* than in TFA and Δ*narG*

Furthermore, 40 genes were found that were expressed similarly in the WT strain and in Δ*narG* mutant, but induced in Δ*fnr* mutant. These could be the genes actually repressed by Fnr in anaerobiosis. However, not all of them showed repression in anaerobic conditions in the WT strain, being some of them even induced in these conditions.

Belonging to this group, the membrane protein gene SGRAN_1383 was expressed to very low levels in aerobiosis and anaerobiosis in TFA and in anaerobiosis in the Δ*narG* mutant in our dRNA-seq assays, but was induced 50 fold in anaerobiosis in Δ*fnr*. However, induction kinetics by RT-qPCR (Figure 35) showed that this gene was actually induced up to 20 fold in TFA in anaerobic conditions in 2 hours, but its expression then decreased to reach aerobic levels of expression by the time the samples for the dRNA-seq where taken, which explains why no induction of this gene in anaerobic conditions was initially detected. This gene was anaerobically induced in TFA in a similar way regardless the presence of nitrate, while in anaerobic conditions with nitrate in Δ*narG* it was not induced. However this gene reached its highest induction in

Δ*fnr* mutant in anaerobic conditions with nitrate, when it was induced up to 65-fold in 4 hours. The phenotype of this gene is difficult to explain, but one hypothesis is that this gene could at least temporarily be induced by anoxic conditions in an Fnr-independent way, but that Fnr could be actually repressing its expression when becoming active.

Other genes showing similar pattern of expression are SGRAN_1677, encoding for an uncharacterised protein, which was induced 11-fold in Δ*fnr*, although it was not repressed in anaerobiosis in TFA, and SGRAN_3431, encoding for a membrane protein, which was induced 20-fold in Δ*fnr* mutant but slightly repressed in anaerobiosis in the WT. Once again, the behaviour of these genes is not easy to explain.

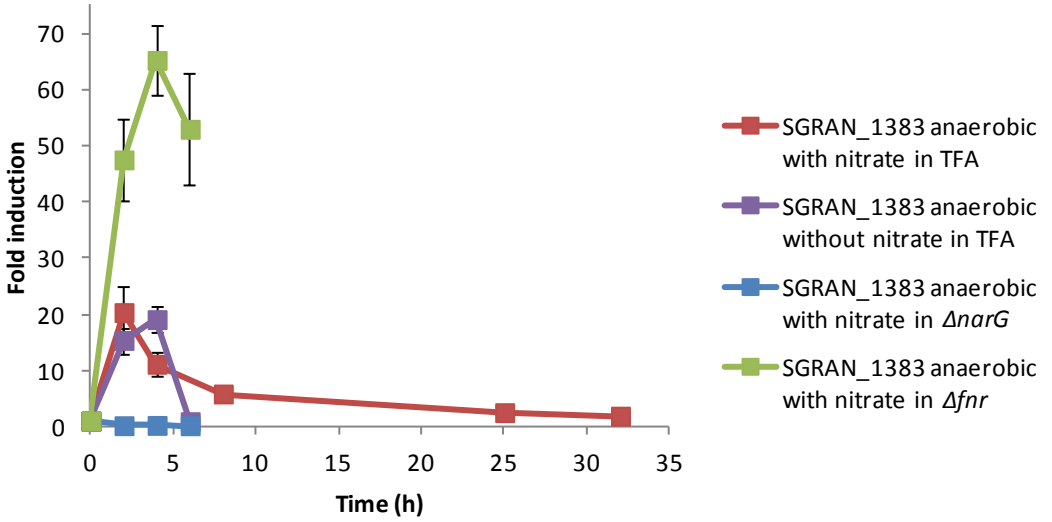


Figure 35. Induction kinetics of SGRAN_1383. Anaerobic conditions with 20 mM nitrate in the WT strain are represented in red, anaerobic conditions without nitrate in the WT strain in purple, anaerobic conditions with nitrate in the Δ*narG* mutant MPO253 in blue and anaerobic conditions with nitrate in the double Δ*fnr* mutant MPO252 in green. Fold change induction over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

<u>Genes much more expressed in Δ*fnr* and Δ*narG* than in the WT that are not induced in anaerobiosis</u>

Finally, 41 genes were found that had very low expression in the WT strain in all conditions, including anaerobiosis, but were more expressed in Δ*fnr* and Δ*narG* mutants, being all these genes at least 14-fold more induced in Δ*fnr* than in the

WT strain. The 24 most induced genes in Δ*fnr*, that were also highly induced in Δ*narG*, coded for transposases or proteins involved in transposition or integration of phages and plasmids.

Among the transposases belonging to this category, there is a pair of them that is in triplicate in the genome, SGRAN_1432-1431, SGRAN_2502-2501 and SGRAN_3210-3211, three almost identical copies, being just SGRAN_3211 shorter and with some differences in the 3' terminal part compared to SGRAN_1431 and SGRAN_2501. By RT-qPCR (Figure 36), using primers that annealed with the three genes SGRAN_1432, SGRAN_2502 and SGRAN_3210, we observed that these genes were induced very fast in the WT strain in anaerobic conditions with nitrate, up to 8-fold in 2 hours, but this induction was subsequently lost, which again explain why these genes were not detected as anaerobically induced. Induction of these genes was actually higher in TFA (8 fold) than in the mutants. In anaerobic conditions without nitrate in the WT strain and in anaerobic conditions with nitrate in Δ*fnr* these genes were slightly induced, not being induced in Δ*narG* mutant in anaerobic conditions with nitrate. This suggests that these genes could be quickly responding to the change to anaerobic conditions, and then this repression is reverted when cells are adapted to anaerobic growth. However, this response seems to require respiration of the bacteria since induction in TFA was less in the absence of nitrate (Figure 36).

Some of the genes belonging to this group, however, were also induced in another condition: *ppsA* PEP synthase was induced by NO and the TetR-like regulator SGRAN_1050 was induced 12-fold by carbon starvation.

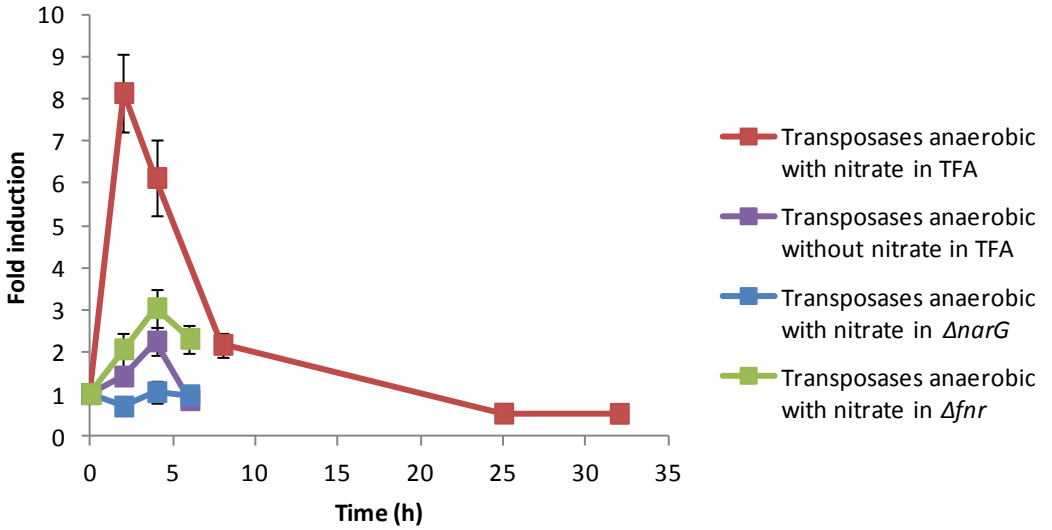


Figure 36. Induction kinetics of transposases much more expressed in Δ*fnr* and Δ*narG* than in the WT that are not induced in anaerobiosis. Fold induction of transposases SGRAN_1432, SGRAN_2502 and SGRAN_3210 in anaerobic conditions with 20 mM nitrate in the WT strain (red), anaerobic conditions without nitrate in the WT (purple), anaerobic conditions with nitrate in the Δ*narG* mutant MPO253 (blue) and anaerobic conditions with nitrate in the double Δ*fnr* mutant MPO252 (green). Fold change induction over time with respect to time 0 is shown and graphics represent the mean ± SD of 3-4 technical replicates.

3.3.2. Fnr box identification

In order to determine the consensus sequence, the Fnr box for FnrN and FixK to which they would bind to regulate the expression of their target genes, we selected genes expressed more than 4-fold in the Δ*narG* MPO253 mutant as compared to the Δ*fnrN*Δ*fixK* MPO252 double mutant, and analysed their upstream regions using the bioinformatic tool MEME, which looks for conserved patterns among the provided sequences. We then looked for the sequence pattern obtained in all the genes analysed in the first instance, using the bioinformatic tool FIMO. The genes that showed this consensus sequence were once again analysed using MEME to obtain a more accurate Fnr box for TFA, which can be seen in Figure 37.

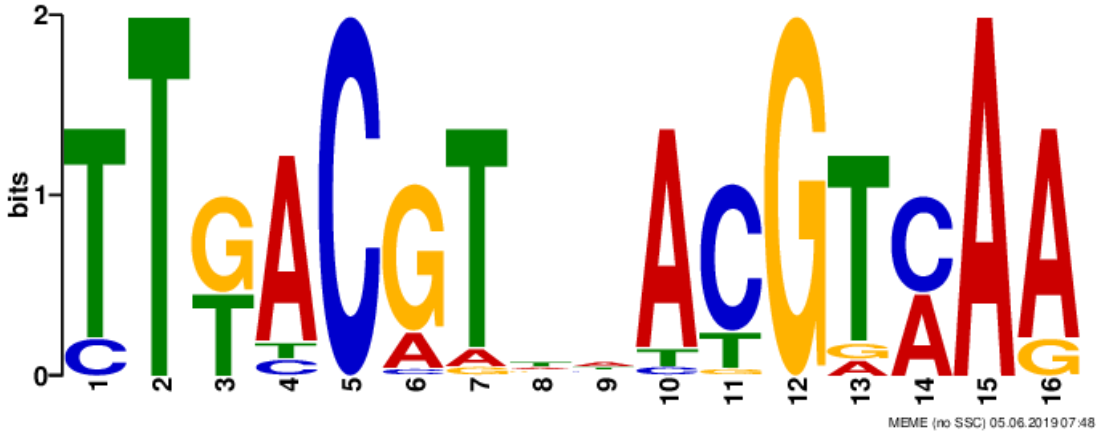


Figure 37. Consensus for FNR box in TFA. Diagram showing the consensus binding sequence for Fnr in TFA obtained using the tool MEME.

Although there was a small variability in some of the bases, the two most common consensus sequence for Fnr box in TFA were TTGACGTN$_2$ACGTCAA and TTTACGTN$_2$ACGTAAA.

The search of the consensus sequences found in the upstream region of the genes suspected to be regulated by FnrN and FixK according to the dRNA-seq analysis allowed us to define the direct Fnr regulon in TFA. We have found 14 operons in TFA that show Fnr boxes in the upstream regions of their first genes, which are shown in Table 10.

SGRAN number	Name	Description	Operon
0602	*cybB*	Cytochrome b_{561}	-
2448	*ompW*	Outer membrane protein W	-
2449	*ccoN*	Cytochrome *c* oxidase polypeptide I-like protein	SGRAN_2449-2458
2714	*ilvX*	Thiamine pyrophosphate-dependent enzyme, possible carboligase or decarboxylase	-
3353	*uspA*	Universal stress protein UspA	-
3423	*etfB*	Electron transfer flavoprotein subunit beta	SGRAN_3423-3422

3802	EBMC1_01250	Cupin	SGRAN_3802-3800
3843	BAY1663_04176	Uncharacterised protein	-
3845	*narU*	Nitrate/nitrite transporter NarU	SGRAN_3845-3856
3856	*moeA*	Molybdenum cofactor biosynthesis protein MoeA	SGRAN_3845-3856
3858	*yhbT*	Uncharacterised protein	SGRAN_3858-3860
3861	*fixK*	Nitrogen fixation regulation protein FixK.	-
3862	*nrdZ*	Ribonucleotide reductase	-
4065	*acdA_2*	Acyl-CoA dehydrogenase	-

Table 10. TFA genes with predicted Fnr boxes. The SGRAN number, name and description of the genes with a predicted Fnr box in their upstream region are shown.

Genes that seem to be regulated by Fnr proteins, shown in Table 10, belong to different functional categories. There are genes that may be helping TFA to adapt to these new conditions, as the *ompW* gene, that in *E. coli* codes for a porine involved in metabolic transitions in anaerobiosis and that is regulated by Fnr[336]. This gene was also induced in anaerobic conditions in TFA, according to the dRNA-seq, so the presence of this Fnr site is in agreement with previous findings. Regarding the gene *nrdZ*, it codes for a O_2-independent ribonucleotide reductase, which belongs to type II ribonucleases that help cells to adapt to anaerobic conditions[337], and is essential for DNA synthesis in anaerobiosis[323]. As expected from the dRNA-seq and RT-qPCR results, *nrdZ* shows a Fnr recognition site in its promoter. The gene *yhbT*, which forms an operon with the genes coding for the proteases YhbU and YhbV, also presents a Fnr box, which is also consistent as we have seen that the genes of this operon were highly induced in anaerobiosis. Moreover, it has been reported, as we mentioned above, that a fast change of the protein profile could be very beneficial for cells when external conditions change, in order to adapt faster to them[338]. This operon has also been reported to play an essential role in UQ biosynthesis in anaerobic conditions in *E. coli*[339].

Several genes involved in response against stress conditions are part of the Fnr regulon. The gene *uspA*, coding for an universal stress protein has also been reported to protect cells against stress situations as well as DNA damage[340], so it is not surprising that in TFA this gene could be regulated by Fnr in anaerobic conditions, as we have seen that this seem to be a stress situation for TFA. However, this gene was induced only 3-fold in anaerobic conditions. SGRAN_3802, coding for a cupin, seems to form an operon with the nitric oxide reductase gene *norB* and with *nnrS2*, being the last also reported to protect cells under NO stress in *V. cholerae* and *S. oneidensis* [341]. Furthermore, these 3 genes were all induced in anaerobic conditions, consistent with the presence of this Fnr box.

There were also several oxidases and cytochrome genes, that showed a Fnr box. As expected, we have found an Fnr box upstream *narU*, the first gene of *nar* operon that encodes the nitrate reductase components, and that is induced in anaerobiosis. Furthermore, upstream from the gene coding for the molybdenum cofactor biosynthesis protein, *moeA*, the last gene of the long *nar* operon, an Fnr box was also detected, located inside the sequence of the previous overlapping gene *moaC*. So there appears to be an internal promoter within this long operon, probably to guarantee sufficient expression of the last gene *moeA*.

The *cco* operon, coding for a cytochrome *c* oxidase, also bears an Fnr box, which is consistent with the fact that these oxidases have been reported to have high affinity to oxygen, thus allowing respiration when oxygen concentrations are very low[22,23]. However, this is in contrast with the fact that in the dRNA-seq analysis the first genes of the operon were barely affected in anaerobiosis, being induced only from *ccoH* on.

Belonging to this group we also found the cytochrome b_{561} gene *cybB*, and *etfB*, the first gene of the *etfBA* operon, coding for an electron transfer flavoprotein essential for anaerobic carnitine reduction in *E. coli*[342], which is surprising as none of them is affected in anaerobiosis.

It is interesting that the *fnr* anaerobic regulatory gene *fixK* also presented an Fnr box, being likely regulated by its own protein and/or by FnrN, which is

consistent with the fact that this gene is induced in anaerobic conditions. On the other hand, we have not found an Fnr box upstream *fnrN*, thus suggesting that these two genes are regulated differently, which could also have repercussion on their function.

We have also found Fnr boxes upstream the Acyl-CoA dehydrogenase gene *acdA_2*, in the possible carboligase or decarboxylase *ilvX* and in the uncharacterised gene SGRAN_3843, although their biological significance in these conditions is not still clear.

The Fnr boxes of most of these genes (*cybB*, *ccoN*, *uspA*, SGRAN_3802, SGRAN_3843, *narU*, *yhbT*, *nrdZ* and *acdA_2*) were centred in the -41.5 position with respect to the transcription initiation sites previously detected[343], thus being these class II Fnr-activated promoters, the most common ones[94]. Regarding the genes *ilvX* and *etfB*, they showed the Fnr box a little further away, centred in positions -50 and -53, respectively, but none of these genes were affected in anaerobic conditions, maybe because of the displaced position of these Fnr sites. Regarding the *moeA* gene, there is an Fnr site very far way, centred in position -146 with respect to an internal TSS detected within the upstream *moaC* gene. This gene was induced in anaerobic conditions, but its induction level was very similar to that of the upstream genes of the operon, suggesting that this expression may be consequence of the presence of the Fnr box at the beginning of the *nar* operon and not of its own Fnr box. Finally, the Fnr boxes of the genes *ompW* and *fixK* were located 161 and 78 nucleotides upstream of their start codons, respectively, but we have not localised in our analyses the TSS of these two genes, so we do not still know in which positions with respect of them are their Fnr boxes located.

4. Flagellar expression and motility in aerobic and anaerobic conditions in TFA

Bacterial flagellum is a complex system, formed by external component outside the cell wall, such as the filament, the hook, and the hook-associated proteins, and internal components, which consist of rings and several auxiliary proteins that are able to rotate and transfer this rotation to the filament, thus allowing the

bacteria to move[200,201,208]. Therefore, the complete flagellar system is encoded by a high number of genes, depending on the bacteria.

As previously mentioned, TFA has 39 putative contiguous flagellar genes (SGRAN_4088-4126), apparently distributed in 4 operons, including a putative σ^N dependent activator, a *fliA* gene coding for σ^{28} and its anti-sigma *flgM* and two genes, *fliC* and *fliC2*, coding for flagellins. According to the RNAseq, all of these genes were repressed in anaerobic conditions. The gene arrangement together with the repression extent in anaerobiosis is shown in Figure 38. Since this phenomenon had not been reported before, we considered interesting to study in more detail the motility and flagellar production of this bacterium in aerobic and anaerobic conditions.

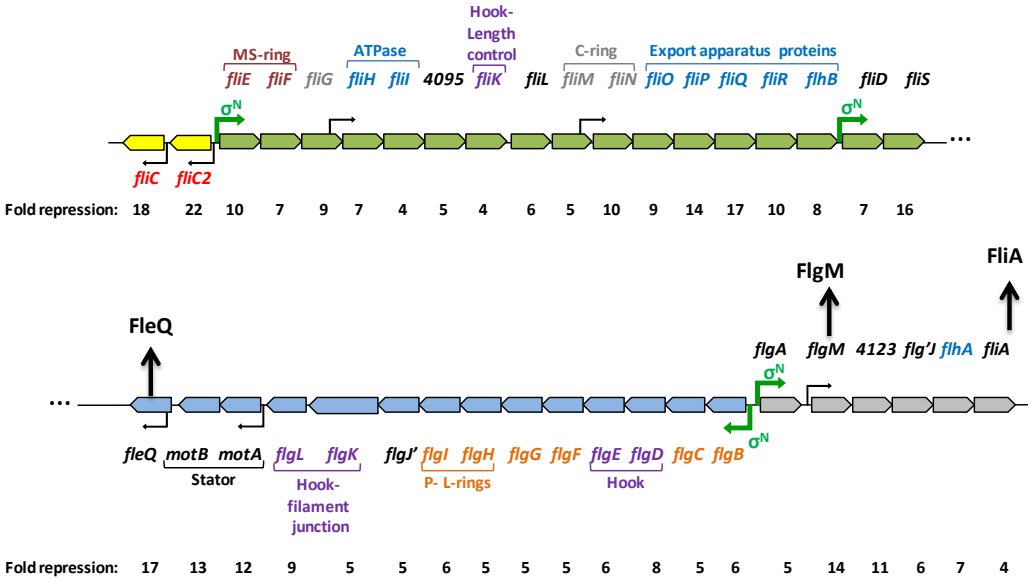


Figure 38. Flagellar genes of TFA. Here we show the flagellar operons found in TFA with their primary and internal promoters. The different flagellar components for which they code are also indicated. Underneath each gene, their repression fold in anaerobic conditions is shown.

Among these flagellar operons, SGRAN_4107, putatively coding for a σ^N dependent activator, was initially annotated as *nifA6*. However, this gene, which lacks the N-terminal regulatory domain, also showed similarity to the FleQ flagellar regulator of *P. aeruginosa*, particularly, a 33% identity in the common domains (Figure 39). The localisation of this gene among all the flagellar genes, the homology with the FleQ protein and the fact that a number of σ^N dependent

promoters appear to drive transcription of many flagellar genes (Figure 39), made us hypothesise that this protein could be the activator of flagellar genes, so we designated it FleQ.

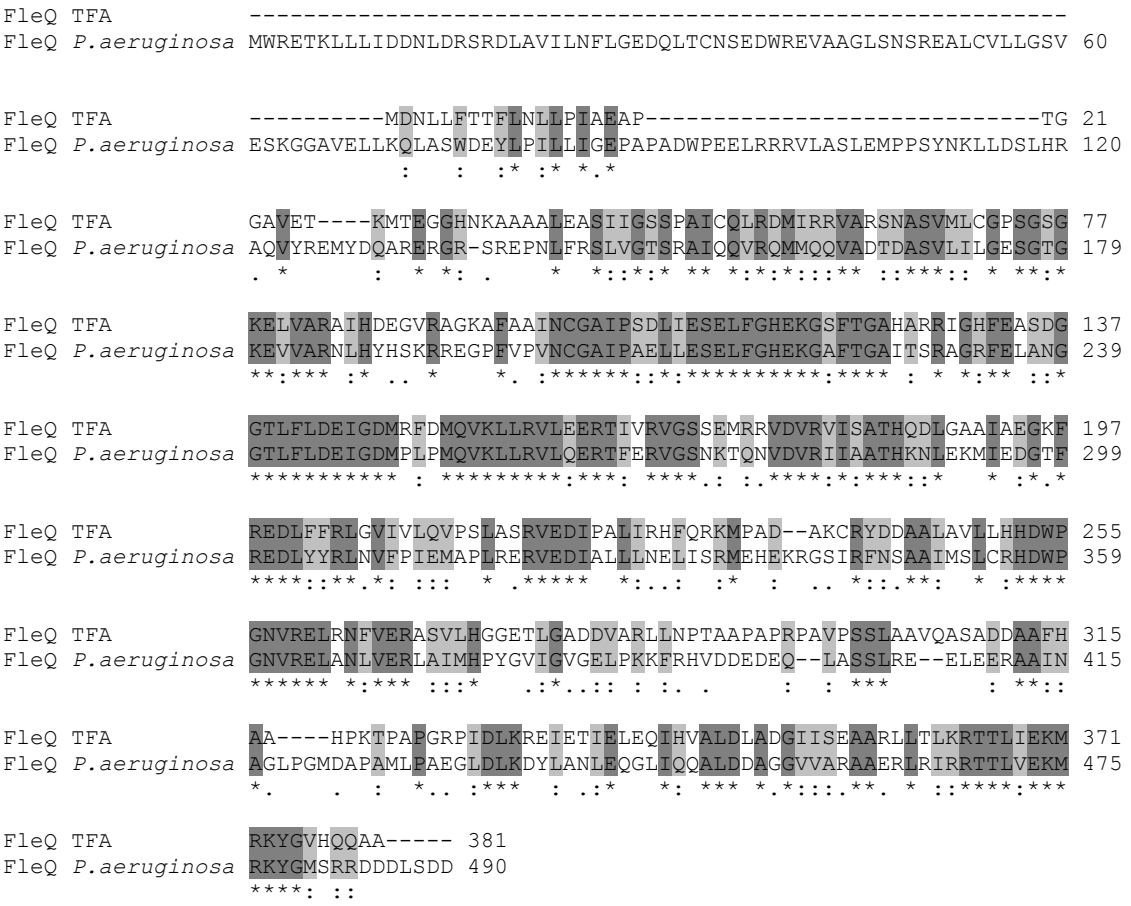


Figure 39. Aligment of SGRAN_4107 protein product with the FleQ of *P.aeruginosa*. The putative FleQ protein of TFA, coded by SGRAN_4107, was aligned with the FleQ protein of *P. aeruginosa*, using the Clustal Omega program. Identical residues are highlighted in dark grey and marked with an asterisk while similar residues are highlighted in light grey and marked with colon.

4.1. Motility and flagella detection by electron microscopy in WT strain

As we have seen in the transcriptomic results that flagellar genes were downregulated in anaerobic conditions in contrast to aerobic conditions, we wanted to determine if TFA motility was also reduced in anaerobic conditions. For that purpose, we performed swimming motility assays in both aerobic and anaerobic conditions.

First of all, to determine the best conditions for swimming assays, we tested swimming in aerobic conditions in 0.3% agar plates with rich MML medium or mineral medium with β-HB 40 mM, by puncturing the cell biomass inside the agar or leaving this biomass on the surface of it (non-punctured). Our results showed that the largest swimming circles were achieved when cells were punctured in mineral medium plates (Figure 40). Therefore, we decided to keep this condition for the subsequent swimming assays.

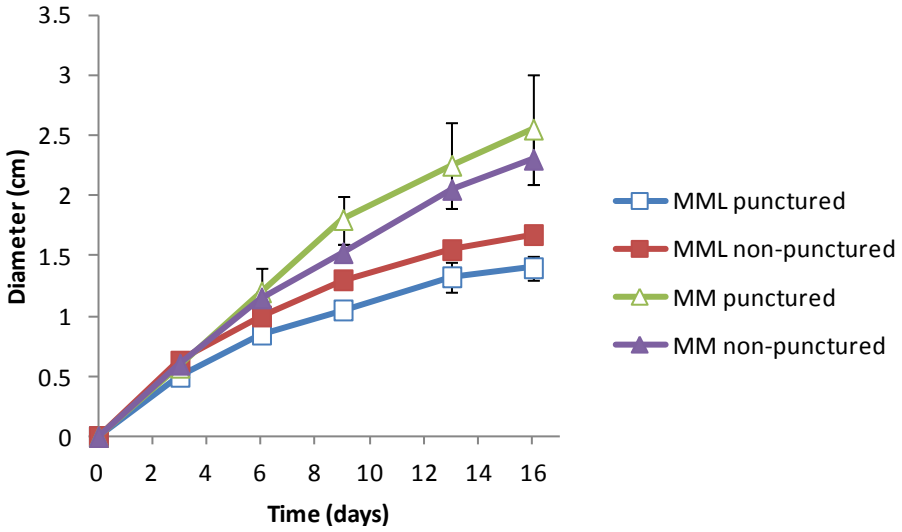


Figure 40. Swimming assays of TFA in different conditions in aerobiosis. The diameters of the swimming circles are shown here with respect to time for TFA in aerobic conditions in rich MML (squares) and mineral medium with β-HB 40 mM (triangles). Cell biomass was either punctured inside the agar (empty symbols) or put in the surface of the agar (filled symbols). Graphics represent the mean ± SD of 2 biological replicates.

During these swimming assays, a few protuberances were formed in some swimming halos of the wild type, which suggested that motility of the bacteria in this part of the halos was higher. Four spontaneous mutants that showed higher swimming motility and formed bigger halos were isolated from the biomass of these protuberances (Figure 41).

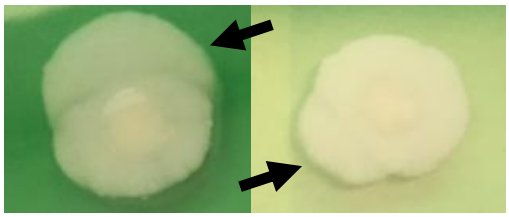


Figure 41. Spontaneous swimming mutants isolation. Swimming circles formed by TFA from which faster swimming spontaneous mutants were isolated from the protuberances pointed by the arrows.

Once the best conditions for the swimming assays were established, new assays in anaerobic conditions with nitrate 20 mM as final electron acceptor and in aerobic conditions were performed with TFA and with one of the faster swimming spontaneous mutants, MPO255. As defined above, these experiments were performed in mineral medium plates with β-HB 40 mM and puncturing the cell biomass inside the agar. We observed that in anaerobic conditions TFA was barely able to swimming compared to aerobic conditions. It could also be seen that MPO255 swam faster that the wild type strain and formed bigger swimming circles, both in aerobic and anaerobic conditions (Figure 42).

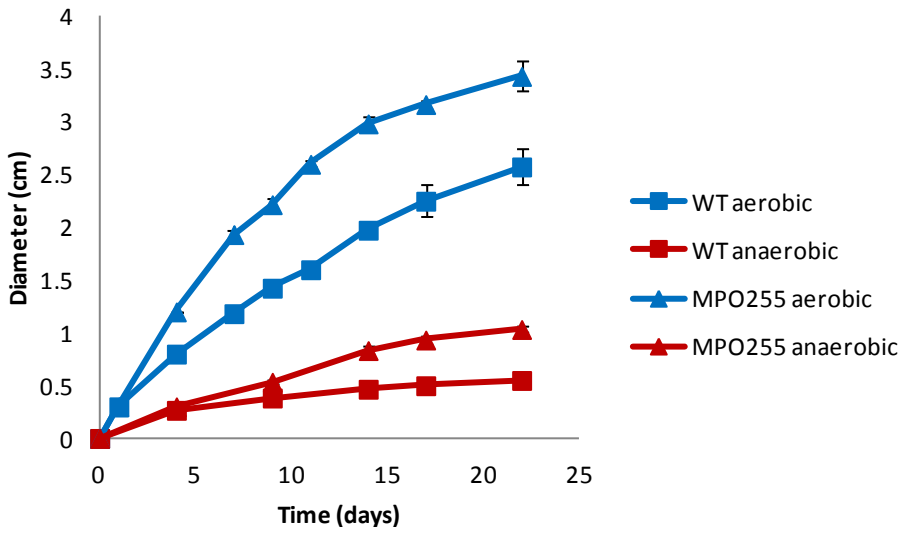


Figure 42. Swimming assays in aerobic and anaerobic conditions of TFA and the faster swimming mutant. The diameters of the swimming circles here along the time are shown for TFA (squares) and the faster swimming mutant MPO255 (triangles) in aerobic conditions (blue) and anaerobic conditions (red) in mineral medium with β-HB 40 mM (triangles). Graphics represent the mean ± SD of 2 biological replicates.

Since swimming circles are formed by a combination of growth and motility of the bacterial progeny, in order to see if this bigger swimming circles of MPO255 with respect to the wild type strain could be a consequence of a faster growth of the MPO255 mutant, we performed aerobic growth curves both in rich MML medium and in MM medium with β-HB 40 mM comparing TFA to MPO255. Results showed that MPO255 did not present significant differences in growth with respect to the wild type strain in neither of the media (Figure 43), therefore indicating that the difference in the swimming circles is not due to differences in growth.

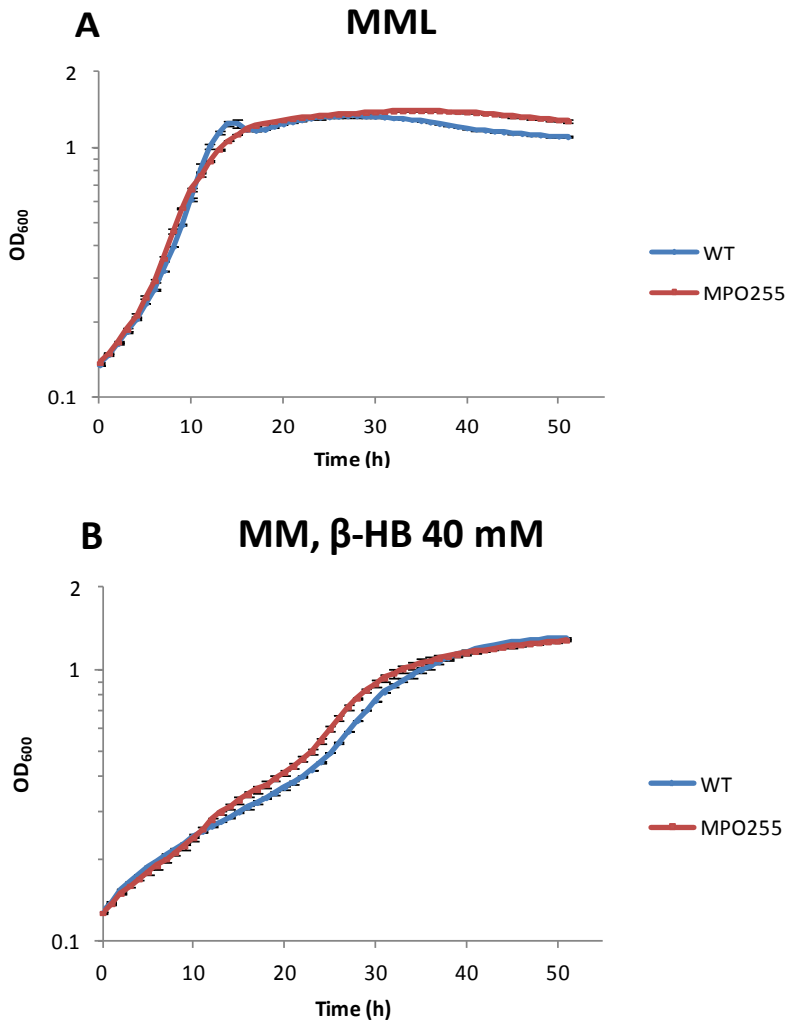
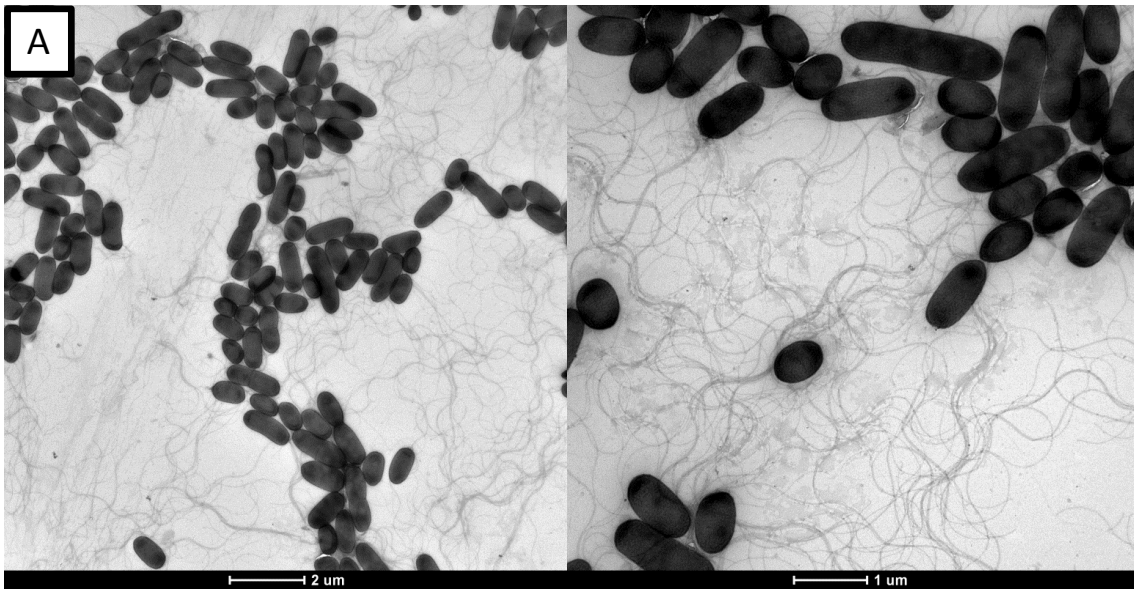


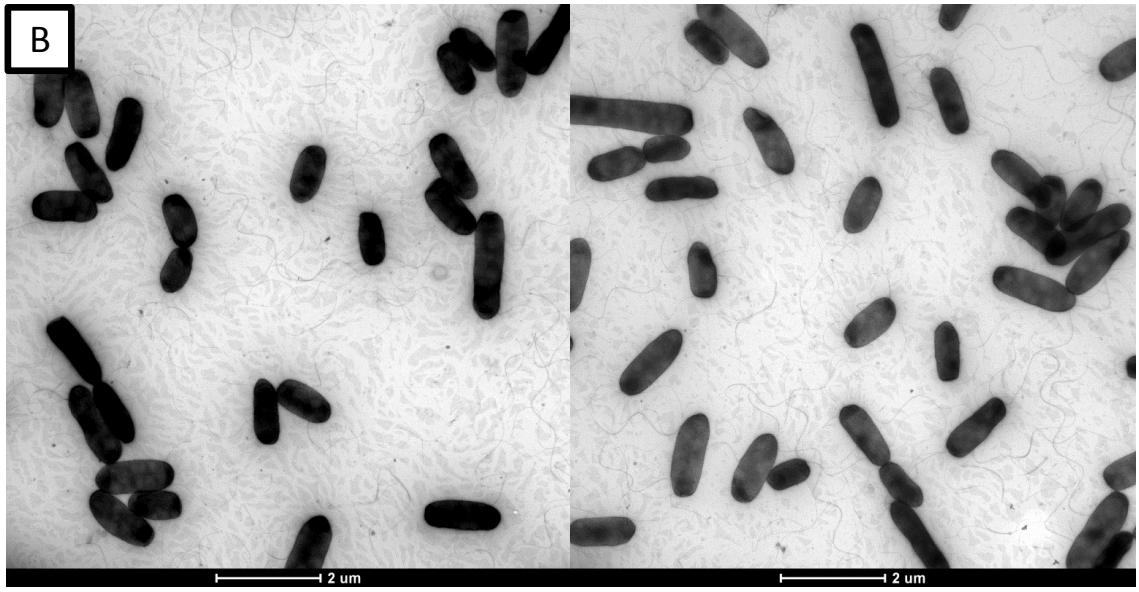
Figure 43. Aerobic growth of the faster swimming mutant with respect to TFA. Aerobic growth, represented as OD_{600} over time, in rich medium MML (A) and mineral medium with β-HB (B) is shown for TFA (blue), and the spontaneous faster swimming mutant MPO255 (blue). Graphics represent the mean ± SD of 3 biological replicates.

Results

Subsequently, in order to characterise TFA flagella and to see if anaerobic conditions led to an absence of flagella, as suggested by the transcriptomic analyses, we performed electron microscopy of TFA in aerobic and anaerobic conditions, in collaboration with the group of Dr. Jos van Putten in Utrecht (The Netherlands). Cells were taken both from plates or from liquid cultures in minimal medium with β-HB 40 mM and nitrate 20 mM for anaerobic conditions.

Figure 44 shows that in aerobic conditions (A and C) flagella can be clearly observed in the samples and their number was high, while in anaerobic conditions (B and D) flagella are barely detected. This suggests, as expected, that TFA does not produce flagella in anaerobic conditions, being the few flagella observed in the images probably the remaining from the previous growth in aerobic conditions. In aerobiosis, the images taken from cells coming from plates (A and B) appeared cleaner than those taken from cells from liquid cultures (C and D). However, it seems that TFA flagella detach very easily from the cells with manipulation, so the samples from liquids cultures (C and D), in which manipulation was softer, allowed us to see flagella attached to the bacteria. In the resulting images, at larger magnification (E), it appears that TFA has a polar bunch of flagella which is surrounded by a polar bunch of fimbriae. It was not possible to determine the number of flagella present in TFA due to the fact that they seem to detach very easily, as mentioned. Neither the bunch of flagella nor the bunch of fimbriae attached to a cell pole could be observed in anaerobic conditions.

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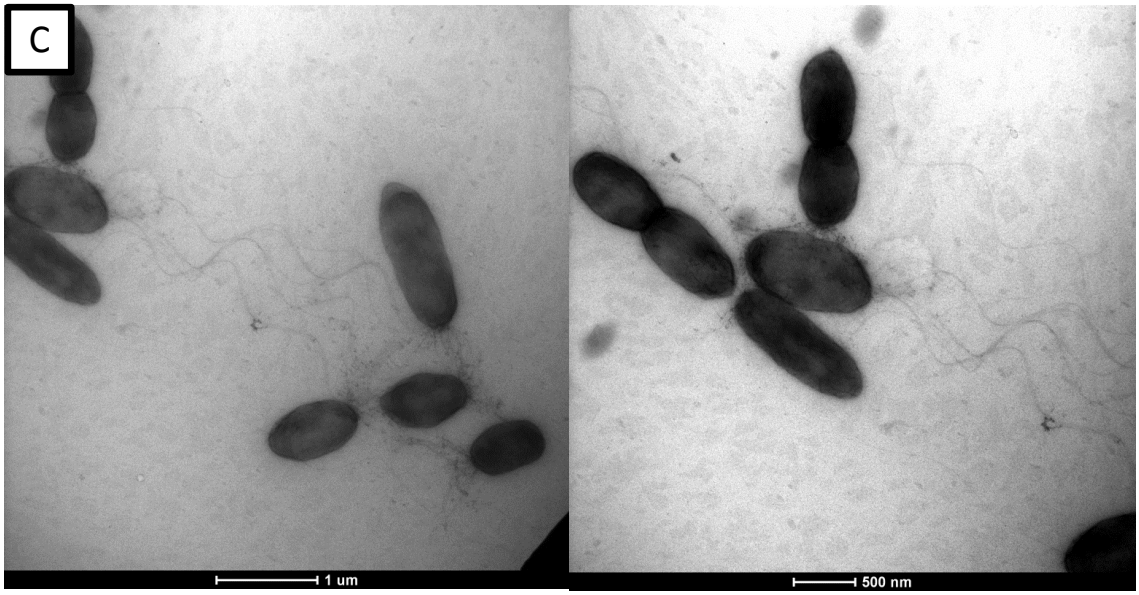
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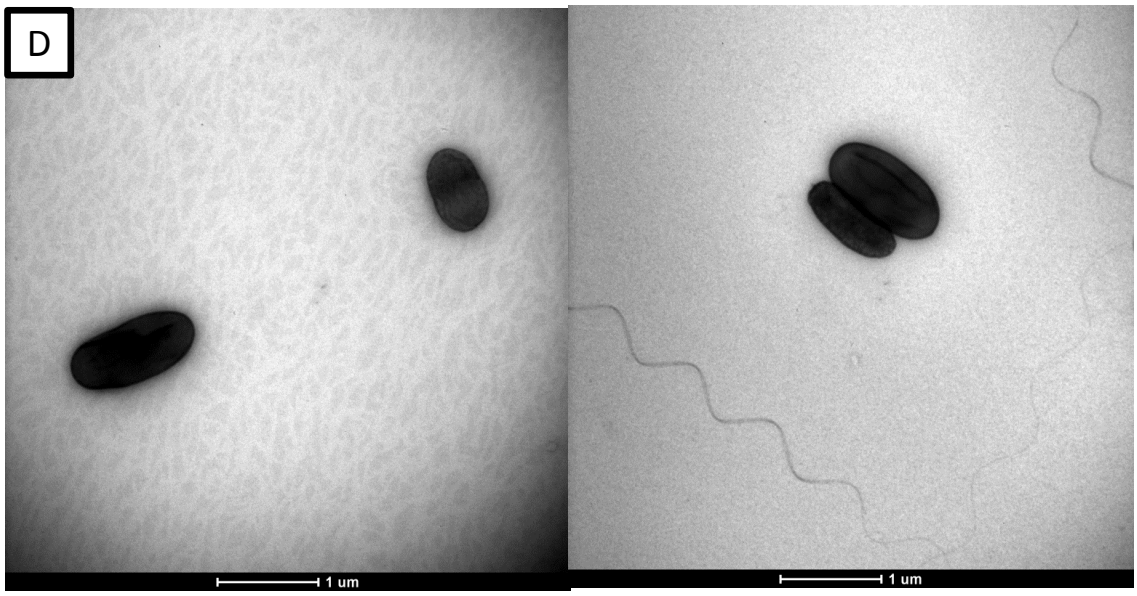
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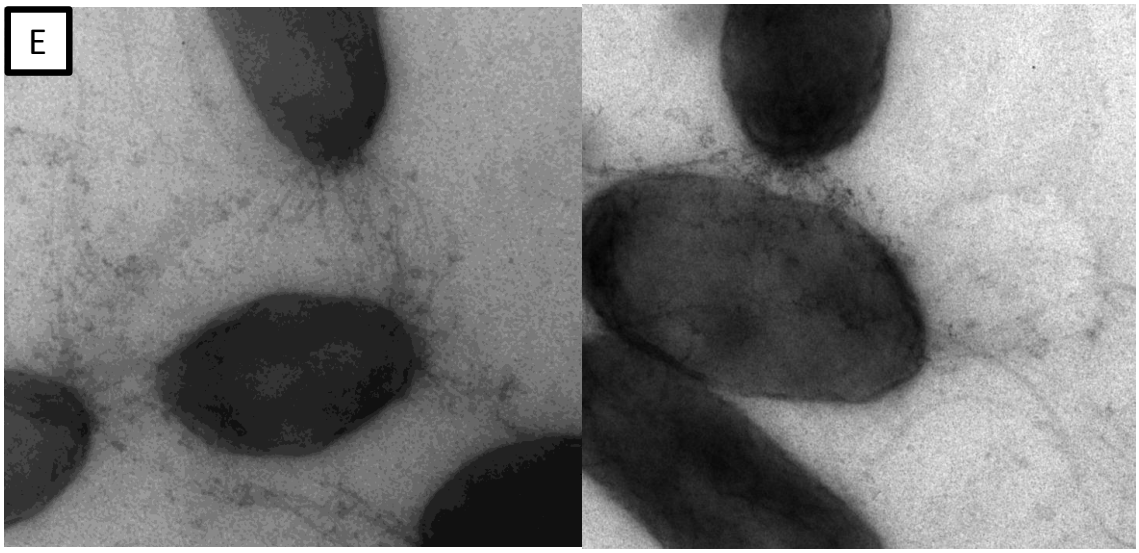


Figure 44. Electron microscopy of TFA in aerobic and anaerobic conditions. Images were taken from cell in aerobic (A and C) and anaerobic (B and D) conditions in minimal medium with β-HB 40 mM and nitrate 20 mM for anaerobic cultures. Images A and B correspond to cells from plates while images C and D correspond to cells coming from liquid cultures. Panel E shows amplified sections of the images in panel C in which flagella and fimbriae can be better observed.

4.2. Characterisation of the regulators CtrA, FleQ and FliA

As previously described, the regulation of flagellar genes expression involves several promoters and regulatory proteins with a hierarchical relationship among them. Flagellar genes are classified in 3 or 4 classes, depending on the bacterium, which indicate the order in which these genes are expressed. The regulatory proteins and the genes belonging to each class vary among species[232]. In TFA we have found the putative regulatory proteins CtrA, FleQ and FliA.

In order to determine whether these putative flagellar regulatory proteins CtrA, FleQ and FliA were real flagellar regulators we constructed mutants in their coding genes *ctrA* (MPO256), *fleQ* (MPO254) and *fliA* (MPO851).

Swimming assays were performed comparing the wild type strain, TFA, with the mutants Δ*ctrA*, Δ*fleQ* and Δ*fliA*. These assays were performed in aerobic conditions in mineral medium with β-HB 40 mM 0.3% agar plates and the diameter of the swimming circles was measured over time (Figure 45). Results show that all three mutants were barely able to swim in these conditions in contrast to the wild type strain. These results suggest that these three proteins,

CtrA, FleQ and FliA, are involved in the regulation of motility in TFA, as proposed.

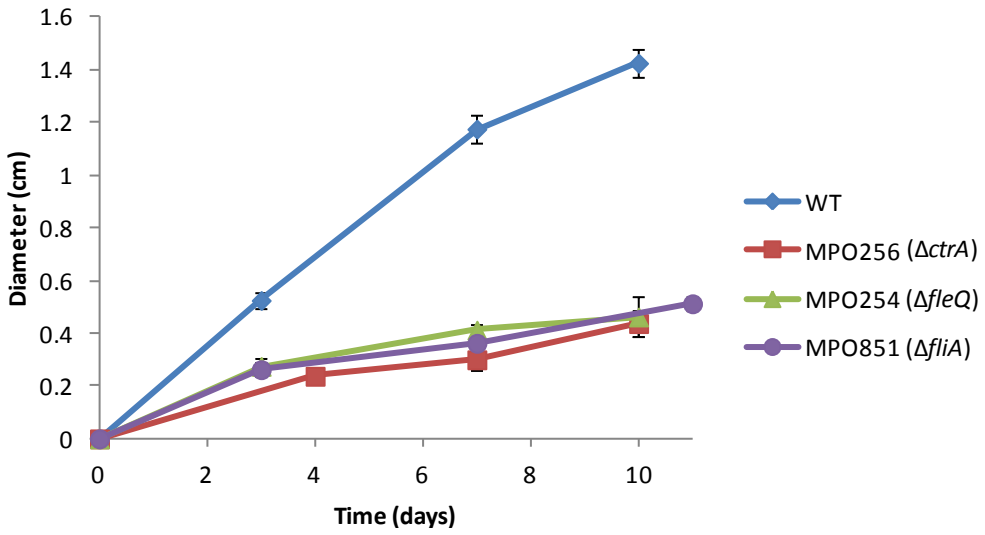
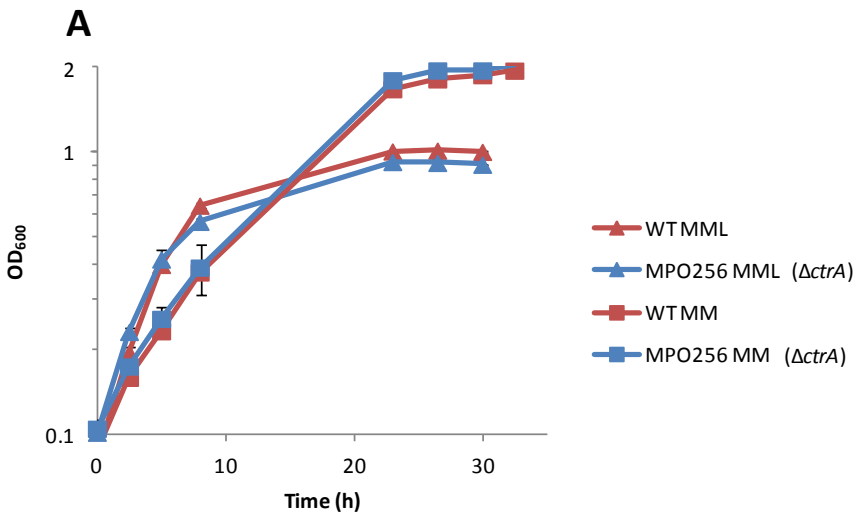


Figure 45. Swimming assays in aerobic conditions of TFA, Δ*ctrA*, Δ*fleQ* and Δ*fliA*. The diameters of the swimming circles are shown here with respect of time for TFA (diamonds), Δ*ctrA* mutant MPO256 (squares), Δ*fleQ* mutant MPO254 (triangles) and Δ*fliA* mutant MPO851 (circles) in aerobic conditions in mineral medium with β-HB 40 mM. Graphics represent the mean ± SD of 4 biological replicates.

To determine whether these smaller swimming circles of the three mutants could be due to a slower growth instead of reduced motility, we performed growth curves of the three mutants in rich medium and mineral medium with β-HB, and compared to the wild type strain (Figure 46). The mutants did not show significant differences in growth in any media, thus indicating that the difference in the swimming circles is not a consequence of differences in growth.



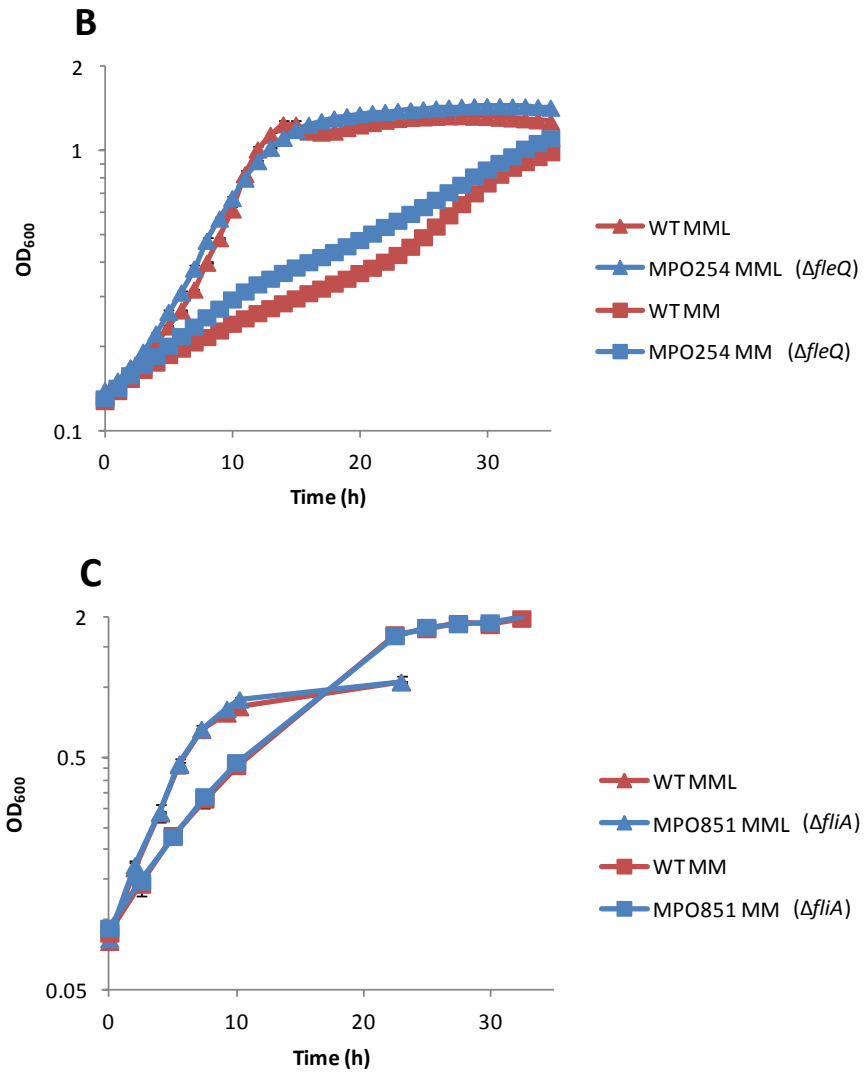


Figure 46. Aerobic growth of Δ*ctrA*, Δ*fleQ* and Δ*fliA* with respect to TFA. Aerobic growth, represented as OD_{600} over time, in rich medium MML (triangles) and mineral medium with β-HB (squares) is shown for TFA (red), and the three mutants (blue) MPO256 (A), MPO254 (B) and MPO851 (C). Graphics represent the mean ± SD of 3-4 biological replicates.

Furthermore, in order to determine whether FleQ could be the protein responsible of regulating the inhibition of flagella in anaerobic conditions we cloned *fleQ* gene in a plasmid under an inducible promoter in order to over-express it, thus yielding plasmid pMPO706. We introduced this plasmid both in the wild type strain and in the Δ*fleQ* mutant MPO254 and we performed swimming assays in aerobic and anaerobic conditions in 0.3% agar plates with mineral medium with 40 mM β-HB of the wild type strain and Δ*fleQ* MPO254 mutant complemented with pMPO706 in the presence and absence of the

inducer IPTG, comparing them to the wild type and MPO254 strains transformed with the empty plasmid pSEVA224 (Figure 47). We observed that in aerobic conditions the over-expression of *fleQ* in the wild type strain had no effect regardless of the presence or absence of IPTG and that the over-expression in Δ*fleQ* MPO254 mutant complemented the mutation, especially when IPTG is added. However, in anaerobic conditions, the over expression of *fleQ* had no effect on MPO254 and only a slight effect in the WT strain when induced with IPTG. These results suggests that the over-expression of *fleQ* is not enough to recover the ability to swim in anaerobic conditions, thus being flagellar regulation in anaerobiosis more complex.

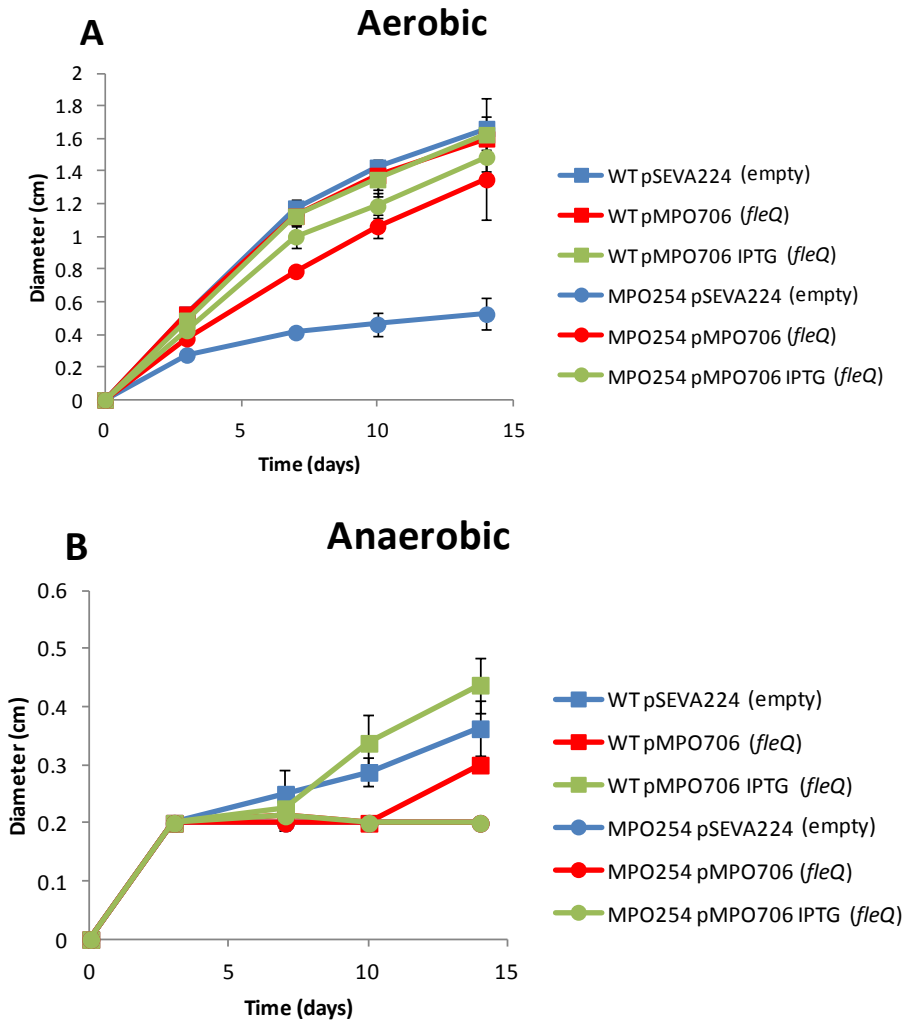


Figure 47. Swimming assays of TFA and Δ*fleQ* mutant complemented with *fleQ*. The diameters of the swimming circles are shown here with respect of time for TFA (squares) and Δ*fleQ* mutant MPO254 (circles), non-complemented (blue), complemented with *fleQ* in pMPO706 (red) and complemented with pMPO706 in the

presence of IPTG (green) in aerobic (A) and anaerobic (B) conditions in mineral medium with β-HB 40 mM. Graphics represent the mean ± SD of 4 biological replicates.

In order to determine if there was flagellar production in TFA in Δ*fleQ*, we performed western blots comparing these conditions in the wild type strain and carrying the WT in anaerobic conditions as a control. For this purpose we used an antibody against the *C. jejuni* flagella and TFA cells grown in minimal medium with β-HB plates, with nitrate 20 mM for anaerobic conditions (Figure 48). Western blots of cell biomass grown under these conditions showed a band of around 35 kDa that could correspond with TFA flagellins, FliC and FliC2, of around 31 kDa each, that appeared in the wild type strain in aerobic conditions but was absent in the wild type strain in anaerobic conditions and in the MPO254 in aerobic conditions. These results suggest the presence of flagella in TFA in aerobic conditions, and its absence in anaerobic conditions and in the Δ*fleQ* mutant MPO254 in aerobic conditions. These results fully agree with the transcriptomic data that showed that flagellar genes were repressed in anaerobic conditions with respect to aerobic conditions, and with the motility assays in Δ*fleQ* mutant.

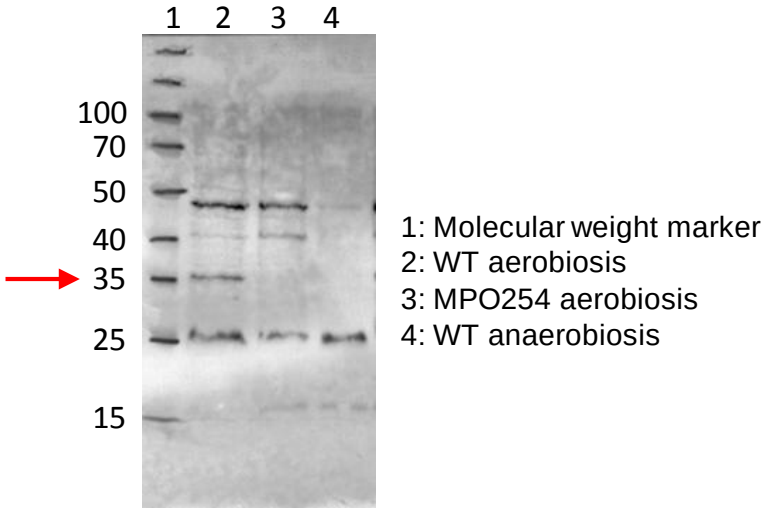


Figure 48. Western blots of TFA in aerobic and anaerobic conditions and the Δ*fleQ* mutant MPO254 in aerobic conditions. Western blot using an antibody against the flagella of *C. jejuni* is shown for TFA in aerobic (1) and anaerobic (4) conditions and for the Δ*fleQ* mutant MPO254 in aerobic conditions (3) in minimal medium with β-HB 40 mM and nitrate 20 mM for anaerobiosis. The molecular weight marker is show in the

first lane (1) and the red arrow indicates the molecular weight in which a band that could correspond to TFA flagellin appears.

in order to see whether the Δ*fleQ* mutant MPO254 had less flagella than TFA or none of them, as expected from the previous results, we performed electron microscopy of this mutant in aerobic conditions using cells from plates and from liquid cultures in mineral medium with β-HB 40 mM (Figure 49). Results show that neither in liquid nor in solid media this bacteria was able to produce flagella and we only observed one individual producing fimbriae, marked in figure 49B with an arrow. This confirms the view that *fleQ* is one of the main regulators of flagellar genes in TFA.

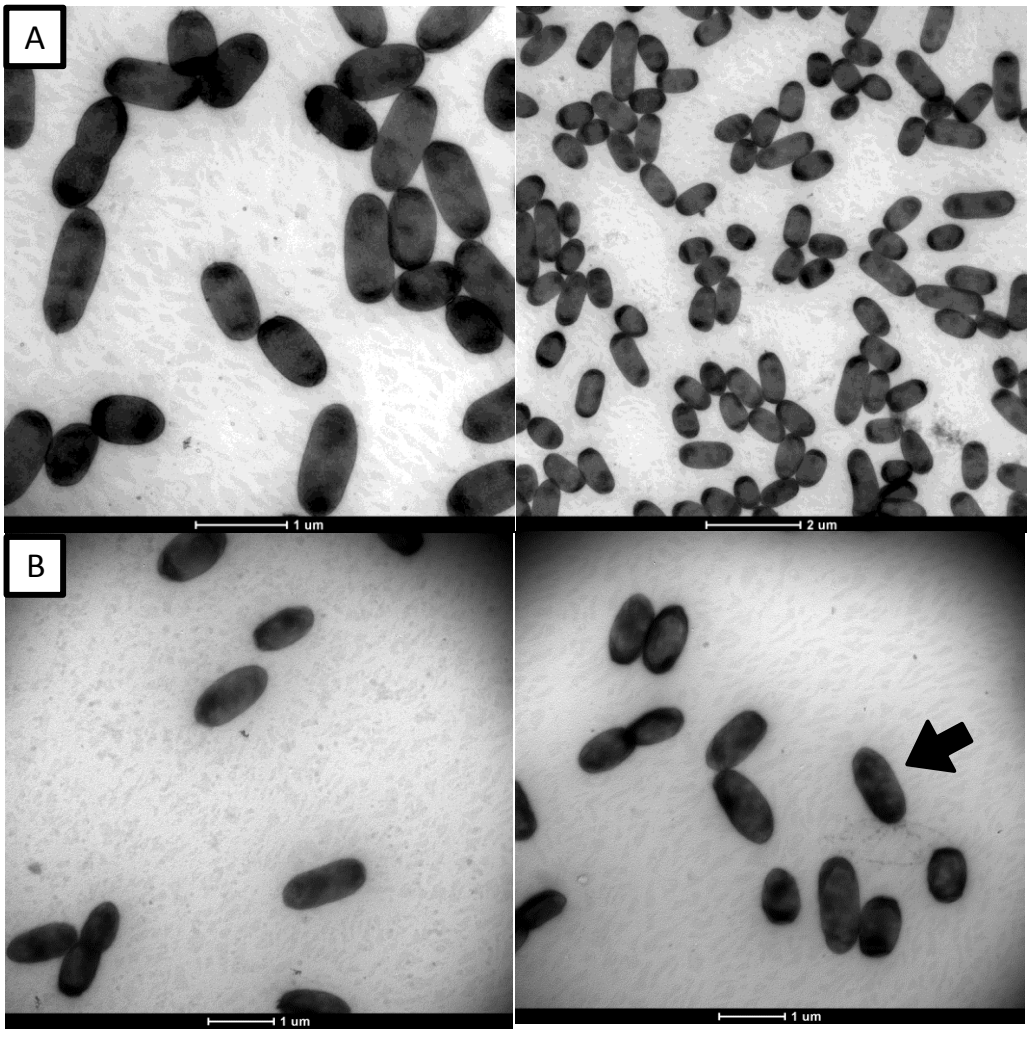


Figure 49. Electron microscopy of MPO254 in aerobic conditions. Images were taken from cells in aerobic conditions in minimal medium with β-HB 40 mM. Images A correspond to cells from plates while images B correspond to cells coming from liquid cultures. The black arrow in B marks a cell found with fimbriae.

Results

Finally, an RT-qPCR comparing the expression level of TFA flagellins FliC and FliC2 in TFA and the Δ*fliA* mutant MPO851 was performed in order to see whether this putative regulatory protein could be regulating the expression of TFA flagellins (Figure 50). The results showed that *fliC* and *fliC2* were 500 ± 80-fold more expressed in the WT strain than in MPO851. This suggests that TFA flagellins expression is actually regulated by FliA, being this protein a flagellar regulator, as expected.

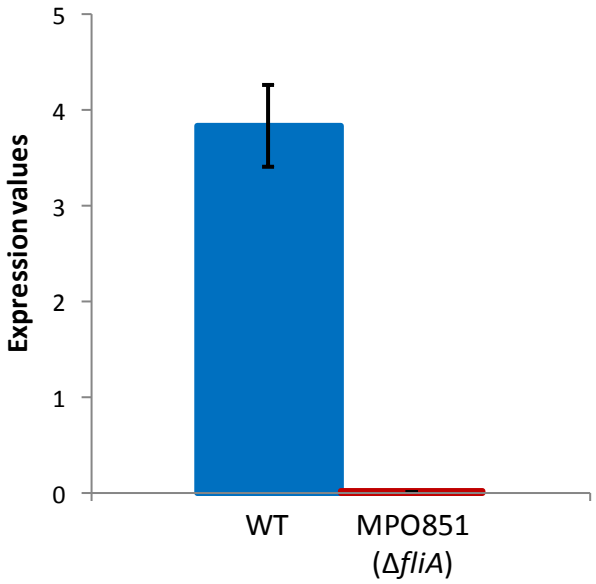


Figure 50. Expression values of *fliC* genes in TFA and the Δ*fliA* mutant. Expression values of *fliC* and *fliC2* genes in TFA and in the MPO851 Δ*fliA* mutant in aerobic conditions, measured by RT-qPCR. These results represent the mean of 4 technical replicates ± SD.

DISCUSSION

Although the genus *Sphingopyxis* has always been described as a group of strict aerobic bacteria[11], the study of the growth of *Sphingopyxis granuli* strain TFA in anaerobic conditions has turn this bacterium in the first reported facultative anaerobic bacterium of its genus[10]. This bacterium has been shown to bear in its genome genes encoding for the nitrate reductase and additional genes required for anaerobic respiration, and to be able to grow in anaerobic conditions by respiring nitrate to nitrite. Two other *Sphingopyxis*, *Sphingopyxis granuli* strain Kw07T and *Sphingopyxis baekryungensis* strain SW-150(T) have been reported as capable of reducing nitrate to nitrate, but not of growing in anaerobic conditions[344,345].

The analysis of the global response of this bacterium to anaerobic conditions has shown differences in the regulation of genes belonging to different functional categories that gives us an insight on the consequences that these conditions and its consequent metabolism have on TFA. We have found particularly interesting the induction of a great amount of genes involved in stress response, detoxification and SOS system and the repression of genes involved in motility and chemotaxis. In the following sections we will discuss these results.

1. TFA growth in anaerobic conditions

As said, TFA is able to respire nitrate to nitrite in anaerobic conditions. This fact could represent an advantage for this bacteria to survive under low-oxygen concentration environments like the mud of the Rhine river where it was isolated[1] or in environments with constant changes between aerobic and anaerobic conditions. Besides anaerobic nitrate respiration, TFA has neither shown the ability to respire anaerobically using any other final electron acceptor or to ferment nor the genes for these activities have been found in its genome.

TFA is able to respire nitrate to nitrite and to excrete this nitrite to the outside of the cell, as we have observed by measuring nitrite and nitrate concentrations in the culture medium during anaerobic growth of TFA. The conversion of nitrate to nitrite has a 1:1 stoichiometry, where one molecule of nitrate is respired to one molecule of nitrite. However, we have observed that the concentration of nitrate consumed was always a few mM higher than the concentration of nitrite

produced, showing up to a 5 mM difference in some conditions. This suggests that part of the nitrite produced is being further transformed into something else, or that part of the nitrate consumed is not respired but transformed into something different to nitrite, which results quite unlikely, since nitrate is the most oxidised form of nitrogen and its reduction would involve nitrite formation in the first step. We have not identified any nitrite reductase coding gene in the TFA genome that could explain further reduction of nitrite. Our interpretation is, therefore, that this difference is a consequence of the nitrate reductase transforming part of the nitrate into nitric oxide during nitrate respiration, as reported in *Salmonella* and other bacteria[102,103]. TFA has also in its genome a gene coding for a nitric oxide reductase, probably involved in nitric oxide detoxification, which is induced in anaerobic conditions. The presence of this enzyme in the apparent absence of an internal source of nitric oxide generation could be explained as a tool to eliminate the nitric oxide that might be produced by other microorganisms with which TFA could coexist in its natural habitat. However, it could also be another evidence that nitric oxide is being produced by TFA.

Regarding anaerobic regulation, we have found two Fnr proteins in TFA, FnrN and FixK. FnrN has proved to be more relevant for anaerobic respiration than FixK, as a mutant in FnrN shows a stronger phenotype and FnrN also complements better than FixK an *E. coli Δfnr* mutant. No other putative anaerobic global regulator has been found in this bacterium. The presence of more than one Fnr type proteins have already been reported in some bacteria like *P. putida*[294], *Burkholderia cenocepacia*[346] and *Herbaspirillum seropedicae*[347]. The three Fnr proteins found in *P. putida* show different sensitivity to oxygen, being one of them highly responsive to low concentrations, while the other two are less sensitive[294]. In *H. seropedicae*, which also shows three Fnr proteins, Fnr1 and Fnr3 have been reported to regulate specific groups of promoters (Groups I and II, respectively), while Group III promoters are putatively co-activated by Fnr3-Fnr1 heterodimers. The function of Fnr2 is still unknown[347]. Regarding *B. cenocepacia*, this bacteria has also 3 different Fnr proteins, though the function of each one has not yet been determined[346]. It would be interesting to study the roles of the two different Fnr

proteins of TFA, to see whether they also have different sensibilities for oxygen or regulate different genes, or, on the contrary, they have no difference in function. The fact that the Δ*fixK* mutant shows a wild type-like growth phenotype suggests that, if each Fnr protein controls different promoters, the ones exclusively controlled by FixK must not be very relevant for anaerobic growth.

We have predicted the sequence of TFA Fnr proteins recognition site, the Fnr box, and shown that the most probable sequences are TTGACGTN$_2$ACGTCAA and TTTACGTN$_2$ACGTAAA, two very similar sequences that just difer in the third position of the palindromes, the ones that are underlined. The first sequence shares high identity with the Fnr boxes reported for other organisms: Fnr of *E. coli* (TTGATN$_4$ATCAA and TTGAGatacATCAA)[140,348], FnrL of *R. sphaeroides* (TTGACatggACTAA)[89], Anr of *P. aeruginosa* (TTGACgtggATCAG)[349], AadR of *Rhodopseudomonas palustris* (TTGATggcgATCAA)[350] and CydR of *Azotobacter vinelandii* (TTGACctgcGTCAA)[351]. The similitude between TFA and *E. coli* Fnr boxes was expected, as we have observed that TFA Fnr proteins were able to complement an *E. coli Δfnr* mutant, thus needing to be able to recognise the *E. coli* Fnr boxes. However, it is still unknown whether these two different Fnr boxes of TFA can be both recognised by FnrN and FixK or each of TFA Fnr proteins recognises more specifically one of the two boxes. Up to the moment we have found 14 operons that seem to have Fnr boxes in their upstream promoter region, though only a selection of genes, the most probably regulated by Fnr due to our dRNA-seq results, was analysed. A more extensive analysis needs to be performed in order to determine the complete Fnr regulon of TFA. However, as we will see below, many genes differentially expressed in anaerobiosis may respond to the consequences of anaerobic growth by respiring just nitrate and not being directly regulated by the anaerobic global regulators.

In our analysis of the global response of this bacterium to anaerobic conditions we have seen, as said, changes in the expression of genes with different functions in the cell. We have observed that the central metabolism of this bacterium was not especially affected in these conditions. However, it is remarkable the high induction levels reached in anaerobic conditions of the

proteases YhbU and YhbV, being two of the most strongly induced genes in TFA in anaerobiosis. These genes seem to form an operon with the gene *yhbT*, operon *yhbTUV*, which was also highly induced in anaerobic conditions. In *E. coli*, curiously, *yhbT* (currently renamed as *ubiT*) is divergent to *yhbU* and *yhbV* (*ubiU* and *ubiV* respectively)[339], so an inversion of this gene must have taken place in TFA to form this operon. Induction of these and other proteases in anaerobic conditions has already been reported, though to a lower extent, in other bacteria such as *E. coli*[352], *Shigella flexneri*[353], *Yersinia intermedia*[354], *P. aeruginosa*[192] and *Corynebacterium glutamicum*[105]. This suggests the importance of a fast change in the protein pattern of TFA when reaching anaerobic conditions from aerobic conditions by actively degrading the proteins that will not be required in these new conditions. This makes especial sense taking into account that anaerobiosis is a energy limiting condition, where a fast reprogramming of the cell protein content could pose a great advantage. Something similar has been reported in *P. putida*, in which genes coding for the components of a putative proteasome where also some of the most highly induced in response to nitrogen limiting conditions, where changes in gene expression were also very drastic[338]. The proteases YhbU and YhbV seem to be directly regulated by Fnr in TFA, as the first gene of this operon, *yhbT*, showed a Fnr box centred in position -41.5 with respect to the transcription initiation site, thus being this probably a class II Fnr-activated promoter, as previously described in *E. coli*[93]. Moreover, these three genes in *E. coli* are essential for the biosynthesis of UQ in anaerobiosis[339], so it would make sense that their expression is induced by Fnr-like activators in TFA if they play a similar role. However, in the dRNA-seq and RT-qPCR experiments with TFA and with the double Δ*fnr* and the Δ*narG* mutants we observed that this operon was only induced in anaerobic conditions when cells were able to grow, thus suggesting that the expression of these genes also requires bacterial growth.

As expected, changes in the respiratory electron transfer chains were observed in anaerobic conditions. The genes coding for the nitrate reductase were induced in anaerobiosis but also other, more unexpected, oxidases (*cyo*, *cyd*, *cco* and *aox*), which use oxygen as final electron acceptor, were induced in these conditions. The first gene of the *nar* operon, *narU*, had a Fnr box, as it

could be expected, centred in -41.5 position with respect to the transcription initiation site, thus likely being its promoter a class II Fnr-activated promoter. Although *nar* operon was induced in anaerobic conditions, this first gene was not apparently induced in anaerobiosis in the wild type strain, because of the high *narU* RNA levels in aerobiosis. However, its expression was lost 30-fold in the Δ*fnr* mutant but not in the Δ*narG* mutant, which indicated an Fnr-mediated regulation. This unusual expression profile could be explained by antisense transcription of *narU*, as up to 6 antisense transcription start sites (TSS) whose transcription levels in aerobiosis higher than those of the rest of *nar* genes have been detected for this gene[343]. We have also observed by RT-qPCR that *narG* was not induced in the Δ*fnr* mutant and barely induced in the Δ*narG* mutant, thus suggesting that, although most probably induced by Fnr proteins, as expected, induction of *nar* operon could additionally require growth or nitrate respiration for its induction. It was very interesting that the last gene of this long operon, *moeA*, coding for a molybdenum cofactor biosynthesis protein, also had a Fnr box upstream of its coding sequence, inside the sequence of the previous gene *moaC*, with which it overlaps, which suggests that there might be an internal promoter to increase expression of the last gene. However, this box is located very far away from a possible internal transcription start site that was detected in aerobiosis[343], actually its position would be centred at -146 from that TSS. In *E. coli*, Fnr boxes centred in positions -61.5, -71.5, -82.5 and -92.5 have been reported, corresponding to class I Fnr-activated promoters. For these promoters, the Fnr proteins located in these boxes are able to contact the C-terminal domain of the RNA polymerase α subunit, thanks to the flexible linker joining the C-terminal domain and N-terminal domain of the polymerase[93]. For genes repressed by Fnr, Fnr boxes at even further positions have been described, as the Fnr box in -106.5 reported for the *narX* promoter in *E. coli*, though in these cases more than one Fnr box is present[96]. Taking into account that this putative Fnr box is even further away than those described, it would be neccesary to perform mutagenesis assays in order to determine whether this is a functional Fnr site or not.

In the case of *cyo* operon, coding for a cytochrome *bd* oxidase, it has been reported these oxidases have high affinity for oxygen, being thus expressed in

oxygen-limiting conditions in some organisms[17]. However, in other bacteria these genes are not induced in the absence of oxygen but are actually even repressed, as it has been reported for *E. coli*[314]. On the other hand, cytochrome *bd* oxidases of *E. coli* have been reported to be essential for respiration in the presence of high H_2S[355]. Apart from their high affinity for oxygen, this fact could also explain *cyo* operon induction in TFA in anaerobiosis as we have seen that in this condition different sulphur detoxification mechanisms were also induced. In addition, the induction of *cyo* has also been reported in *P.aeruginosa* in the presence of the nitric oxide generator S-nitrosoglutathione, which could be another explanation for the induction of this gene in TFA[356]. In our transcriptomic analysis we have seen, however, that the *cyo* genes were induced in aerobic conditions in the presence of NO, though less than 10-fold each, but that *cyoA* was repressed 3-fold in anaerobic conditions with NO. Therefore, the signal or signals that may be inducing the expression of *cyo* operon in anaerobic conditions are still unclear, though these results and the absence of an Fnr site suggest that Fnr proteins are not directly involved in its regulation.

Interestingly, not only anaerobiosis, but also actual nitrate respiration or anaerobic growth was required for the induction of the other 3 different oxidases. The *ccoN* gene, the first of the *cco* oxidase operon, showed an Fnr site in its upstream region, centred at -41.5, thus corresponding to class II promoters. In anaerobic conditions, only the final genes of this operon, starting from *ccoH*, were induced in TFA. Although expression of the proximal genes was not apparently induced in anaerobiosis, its expression was much more drastically reduced in the Δ*fnr* mutant than in the Δ*narG* mutant, thus suggesting that their transcription was actually activated by the Fnr proteins. Lack of induction of the proximal part of the operon in anaerobiosis was apparently due to the unusually high levels of RNA corresponding to the proximal part of this operon in aerobic conditions, as compared to the distal part. As in the case of *narU*, this high transcription of the proximal part detected in aerobiosis could be antisense, as this type of transcription has been detected for *ccoN*, *ccoO*, *ccoP*, *ccoG*, *ccoH* and *ccoI* genes, which would explain this anomalous expression profile.

In the case of the alternative oxidase *aox*, as we have seen previously, these oxidases are insensitive to NO[32]. Considering that we hypothesise that NO is being produced from nitrite in anaerobic conditions by TFA nitrate reductase, that expression of this gene was induced also in the presence of NO both in aerobic conditions and in anaerobic conditions without nitrate, and the fact that *aox* was not induced in anaerobic conditions when there was no nitrate respiration, this oxidase should be likely induced by the NO produced during anaerobic nitrate respiration. Moreover, expression of this gene in the presence of NO and its transcriptional activations by NsrR[33] has been reported in the bacteria *V. fischeri* to help reduce the stress caused by this NO[34], which is consistent with our interpretation.

Regarding the Cyd oxidase, this type of cytochrome *bd* oxidases have also been reported to confer protection against oxidative and nitrosative stress. In the case of oxidative stress, *E. coli* mutants defective in this oxidase showed increased levels of intracellular H_2O_2 and higher sensibility to external H_2O_2[357,358]. It was also observed that the expression of this oxidase was induced when cells were exposed to external H_2O_2[357]. This higher sensibility to H_2O_2 in cytochrome *bd* mutants has also been reported for *A. vinelandii*[359] and *B. abortus*[360,361]. Moreover, *E. coli* cytochrome *bd*-I has been found to have low peroxidase activity[362] and high catalase activity, being the last one insensitive to inhibition by NO[363]. Regarding nitrosative stress, some catalytic intermediates of this enzyme have been reported to bind NO[364-366] and in *A. vinelandii* this enzyme has been reported to oxidise nitric oxide to nitrite[367]. Furthermore, although both HCOs and *bd* oxidases have been reported to have their respiration capabilities inhibited by NO, *bd* oxidases show faster NO-dissociation and therefore lead to a faster recovery of bacterial respiration after NO inhibition when NO disappears[368,369]. Induction of cytochrome *bd* genes in the presence of NO has been reported for *E. coli*[370], *Mycobacterium tuberculosis*[371], *S. aureus*[372], *B. subtilis*[373] and *Desulfovibrio gigas*[374]. The fact that the induction of *cyd* genes in TFA require nitrate respiration could suggest that these genes are induced by the NO produced in anaerobiosis. However, in our dRNA-seq results, none of TFA *cyd* genes were induced in aerobic conditions in the presence of NO.

Discussion

Summarising, from these alternative oxidases genes, only *nar* and *cco* operons seem to be regulated by Fnr and both also require nitrate respiration or cell growth for their full induction. Regarding *cyd* and *aox* operons, both also require nitrate respiration, being *aox* the only one induced by NO. Finally, we do not know how *cyo* is regulated. Therefore, it seems that the different oxidases of TFA induced in anaerobic conditions could be responding to different signals that are generated under anaerobic conditions and we are not sure whether their induction could have functional relevance under these conditions.

The operon *cysJI2*, putatively coding for a sulphite reductase[333], was also induced in anaerobic conditions, losing its induction both in Δ*fnr* and Δ*narG* mutants. This operon could be involved in sulphite anaerobic respiration or just in sulphite detoxification, so it would be interesting to determine if TFA is able to grow anaerobically using sulphite as final electron acceptor.

In addition, there are genes coding for electron transport proteins presumably activated by Fnr but whose behaviour in different conditions is difficult to explain. A cytochrome b_{561} gene, *cybB*, also presented an Fnr site centred at -41.5 regarding its putative TSS. This cytochrome is not induced in anaerobiosis, but its expression was 2-fold lower in a Δ*fnr* mutant but 16-fold higher in Δ*narG* mutant, as compared to the WT strain. In turn, the electron transfer flavoprotein *etfB* present in various anaerobic bacteria[375] also showed an Fnr box, centred at -41.5. This gene forms an operon with *etfA* and both are essential for anaerobic carnitine reduction in *E. coli*[342]. However, none of the *etf* genes was induced in anaerobic conditions in TFA and they were only repressed 4.8-fold in a Δ*fnr* mutant and 2-fold in a Δ*narG* mutant. Interestingly, two antisense TSS have been found in this operon[343] that may explain the low regulation that these genes show in anaerobiosis, despite having an Fnr box.

Regarding cell division and control we have observed the induction of genes such as the O_2-independent type Ib ribonucleotidyl reductase *nrdZ.* This gene showed a perfect Fnr box, centred in -41.5 position, which is not surprising as type II ribonucleases like this one help cells adapt to anaerobiosis[337] and this reductase is essential for DNA synthesis in anaerobic conditions[323]. Accordingly, this gene was induced 69-fold in anaerobiosis and lost this

induction 58.6-fold in the double Δ*fnr* mutant but only 3-fold in the Δ*narG* mutant. All these results suggest *nrdZ* as a perfect candidate to be regulated by Fnr proteins. In *Mycobacterium tuberculosis*, transcription of this gene is depends on DosR/DevR, the primary regulator of the hypoxic response in this bacterium[323]. However, there is not much information about the regulation of this gene. In *P. stutzeri*, other type Ib ribonucleotidyl reductase gene, *nrdD*, is controlled by a Fnr-type regulator, so it is not surprising that in TFA *nrdZ* could be also regulated by Fnr proteins. Our RT-qPCRs also suggest that this gene needs the presence of nitrate for its induction, as it was induced similarly in the WT strain and in the Δ*narG* mutant in anaerobiosis with nitrate but it was not induced in the WT stain in anaerobiosis in the absence of nitrate. We have also detected repression in anaerobiosis of genes involved in cell cycle and cell division regulation, such as *ctrA* and *ccrM*, though no Fnr box was found in any of them. However, induction levels were not reduced for *ctrA* and barely reduced for *ccrM* when growing with tetralin as only carbon source, another slow growth condition, thus suggesting that the repression of these genes in anaerobiosis is not a consequence of slow growth.

We have also observed that many genes coding for ribosomal proteins and other related factors, such us *rplL* and the ribosome recycling factor *frr*, were repressed in anaerobiosis whilst other genes were induced, as the *rsfs* that helps cells adapt to slow growth under restricted nutrient (poor media) and energy (stationary phase) conditions by down-regulating protein synthesis and thereby saving energy. All these data indicate that translation capacity of TFA cells growing under anaerobic conditions is diminished, as expected since it grows more slowly than in aerobiosis.

We have seen the induction of several gene expression regulators involved in regulation of anaerobic metabolism, such as the Fnr type proteins FnrN and FixK and other regulators such as RegAB, FtrB and NsrR. The induction of the Fnr regulators, FnrN and FixK, though expected, was not very high, especially that of FnrN. However it is important to take into account that part of the activation of Fnr proteins in response to anaerobiosis takes places at the protein level, via an structural change and dimerization[91,92], so a basal production of these proteins in aerobic conditions should exist to guarantee a rapid response

to a change of the environment to anaerobic conditions. The *fnr* gene *fixK* of TFA presented an Fnr box, that was identical to that of *E. coli*. This gene was induced 13.6 fold in anaerobic conditions, and lost this expression 81-fold in Δ*fnr* mutant and just 5.4-fold in Δ*narG* mutant. It is interesting that we have not been able to find any Fnr box in the other *fnr* gene of TFA, *fnrN*. This gene was 5.5 fold induced in anaerobic conditions, less than *fixK*, and lost this induction 1300-fold in Δ*fnr* double mutant but did not lose it in Δ*narG* mutant. However, this high repression of both *fnrN* and *fixK* in the double Δ*fnr* mutant is possibly a consequence of the deletion of these two genes in this mutant, and should not be given more attention. Therefore, the results obtained in the dRNA-seq of the WT strain suggest that these two genes are regulated by anaerobiosis, especially *fixK*, which is probably induced by FnrN.

FtrB induction was also expected, as this protein is the homolog of the NarR regulator, responsible of the induction of the nitrate reductase in TFA, as we have seen previously. Moreover, this gene is located upstream of the nar operon and is divergent to it, so it could share the same Fnr box. However, this Fnr box is located very far away from *ftrB* predicted TSS[343], centred in -141, so it is not very probable that this Fnr also works for this gene. The RegAB system is a two-component system that in some bacteria, like *R. capsulatus* and *R. sphaeroides*, is involved in the regulation of anaerobic metabolism, affecting the expression of genes required for photosynthesis, CO_2 fixation, nitrogen fixation, DMSO reductase, hydrogenase and different types of oxidases[376,377]. The induction of this operon is therefore expected, as it may play a role in the regulation of respiration under anaerobic conditions in TFA. Besides, they are part of the *cyo* operon, coding for the cytochrome *o* quinol oxidase, that shows no Fnr box but is induced in anaerobic conditions, as we discussed above. As for NsrR, this is a repressor able to sense nitric oxide via a [2S-2Fe] cluster[134], as explained before, and it was also induced by NO in anaerobic conditions, which supports the hypothesis that TFA is generating NO in anaerobic conditions.

Fnr boxes were also found in the Acyl-CoA dehydrogenase gene *acdA_2*, in the possible carboligase or decarboxylase *ilvX* and in the uncharacterised gene SGRAN_3843. The genes *acdA_2* and SGRAN_3843 were induced to a low

level in anaerobic conditions and lost this induction in Δ*fnr* mutant but not in Δ*narG* mutant. Furthermore, both of these genes presented their Fnr boxes centred at -41.5, thus being likely induced by Fnr. In addition, SGRAN_3843, although uncharacterised, is located next to genes involved in anaerobic respiration, namely *ftrB* (SGRAN_3844) and the *nar* operon (SGRAN_3845-3856), which makes it more likely that it is also involved in anaerobic nitrate respiration. On the other hand, *ilvX* gene was not induced in anaerobic conditions and its expression was repressed just 3-fold both in Δ*fnr* and Δ*narG* mutants. This is consistent with the fact that the predicted Fnr box for this gene is displaced from the described position for Fnr activated genes, not corresponding to either class I or with II promoters positions[93].

We have also seen the induction of a large number of genes involved in stress response and the repression of flagellar, pili and chemotaxis genes, which do not appear to be directly regulated by Fnr proteins, as it will be discussed in the following sections.

2. Anaerobic conditions as a hostile environment for TFA

The induction of the expression of many stress response, detoxification and DNA damage genes suggests that anaerobic conditions are a hostile and mutagenic environment for TFA. Although the induction of some of these genes could be a direct consequence of anaerobiosis, other genes are not induced when the bacteria is unable to respire nitrate. As a result of nitrate anaerobic respiration, a series of nitrate derivatives, such as nitrite and very likely nitric oxide are accumulated in the culture medium. This accumulation of respiration products could be the cause of the induction of these genes that are not induced in the absence of respiration. All this suggests the existence of different stress signals affecting the different stress response genes.

One of these genes is the one coding for the protein YtfE, involved in iron-sulphur cluster repair, suggesting that in these anaerobic conditions TFA iron-clusters are significantly damaged. Nitrosative stress, caused mainly by the accumulation of NO, is one of the most common causes of this kind of damage. Moreover, the *E. coli* mutant in this gene has been reported to be more sensitive to NO and to grow poorly while respiring in anaerobiosis[378]. It is also

remarkable that YtfE induction was slow, as the iron-sulphur cluster damage needs to be accumulated before induction. The apparent co-regulation of genes required for iron-sulphur cluster biogenesis is also consistent with a deficiency of active clusters in anaerobic conditions.

NorB, the nitric oxide reductase detoxification enzyme of TFA, was also induced in anaerobic conditions in TFA, together with other genes that were also induced by NO in aerobic and anaerobic conditions without nitrate. The *norB* gene seems to form an operon with the upstream gene SGRAN_3802, coding for a cupin, and the downstream gene *nnrS2*. The *nnrS* gene has been reported to be regulated by NorR in *V. cholerae*[379], by $FixK_2$ in *Bradyrhizobium diazoefficiens*[380], by NsrR in *S. oneidensis*[381] and by NnrR in *R. sphaeroides*[69]. Furthermore, the heme- and copper-containing membrane protein encoded by *nnrS* has been reported to protect against NO stress in *V. cholerae* and *S. oneidensis*[341], which is also consistent with its co-expression with *norB* and its induction by NO. However, unlike *ytfE*, induction of *norB* was very fast, reaching its maximum induction within the first two hours, which suggest that this operon is induced by NO itself and not by their effects after accumulation. It also suggests that NO production starts very early during anaerobic growth. The fact that upstream of SGRAN_3802 is *nnrR*, encoding for a NO sensing activator of nitrite reductase y nitric oxide reductase genes in denitrifying bacteria[14], also supports the view that this operon is actually induced by NO.

Interestingly, SGRAN_3802, the first gene of this operon, showed an Fnr box in its promoter region, centred in position -41.5. Although the strongest induction of *norB* took place in the first hours, being lower at the time in which the samples for the dRNA-seq were taken, expression of the 3 genes of the operon in the Δ*fnr* mutant was lower than in the Δ*narG* mutant. All this suggests that these genes could also be regulated by Fnr proteins, thus having a dual regulation by anaerobiosis and by NO, although it needs confirmation.

We found very surprising the induction of genes generally involved in oxidative stress. However, the catalase KatA, involved in hydrogen peroxide detoxification, is also involved in protection against anaerobic nitric oxide in *P. aeruginosa*[382] and in *N. gonorrhoeae* many genes responsive to oxidative stress

and/or iron are also responsive to anaerobiosis[106]. All these suggest that, although still unknown, these genes normally involved in oxidative stress could be playing a role under anaerobic stress conditions. We have also observed the induction in anaerobic conditions of all the genes involved in ectoine synthesis. Ectoine is a compatible solute that acts as an osmoprotectant under osmotic stress in bacteria[316]. However, this compound has also been reported to help fight oxidative stress[383]. It has not been reported yet that ectoine can help under nitrosative stress, but we cannot rule out this as a reason for its induction.

The *ompW* gene, coding in *E. coli* for a Fnr-regulated porine involved in metabolic transition in anaerobiosis[336], showed an Fnr site 161 nucleotides upstream its start codon, although we have not yet localised its TSS in our analysis. Our dRNA-seq results showed that this gene was upregulated in anaerobic conditions in the wild type strain, losing this expression in Δ*fnr* mutant. However, its expression was also lost in Δ*narG* mutant, suggesting that its regulation depends also on other factors. The universal stress protein UspA is involved in anti-stress physiological functions and some members of Usp family play a role in the protection of the bacteria against DNA damage[340]. Moreover, it has been already reported that Fnr controls the expression of OmpW and UspA genes in *P. denitrificans*, being both of those proteins downregulated in anaerobic conditions in the Δ*fnr* mutant[384]. In TFA, the *uspA* gene had an Fnr box centred at -41.5, which is consistent with the view that anaerobic conditions are stressful and mutagenic for TFA. However, this gene was just induced 3-fold in the wild type strain in anaerobic conditions, while it was induced in these conditions 7.3-fold in Δ*fnr* double mutant and 5-fold in Δ*narG* mutant, thus showing an induction by a mechanism independent of Fnr, which might be additional to the putative Fnr-mediated activation. For example, the induction of *uspA* in both mutants triggered by this alternative mechanism could be a consequence of the stress generated because of the lack of energy.

This adaptation to anaerobic stressing conditions also seems to affect TFA ribosomes. We have observed the induction in anaerobiosis of the gene coding for RlmH, a protein that, as we previously saw, methylates a residue of the 23 S rRNA which provides fitness advantage under stress conditions[107]. In *E. coli*[385]

and *Shigella flexneri*[353], the homologous gene is not induced in anaerobiosis, but it is induced by other different stress conditions.

Growth in anaerobic conditions led to the induction of the *lexA* repressor, *recA* and two operons coding for error-prone DNA polymerases. The most plausible explanation for the induction of these genes is the emergence of lesions in the DNA that have not been repaired, which supports the view that anaerobiosis seem to also be a mutagenic environment for TFA cells.

In general, the induction of these stress and SOS response does not seem to be regulated by Fnr in TFA, with very few exceptions. In the dRNA-seq of the Δ*narG* and double Δ*fnr* mutants we have seen that the genes that lost their induction in the Δ*fnr* mutant, lost it to a similar or higher extent in the Δ*narG* mutant, suggesting that this lost of the induction is due to the absence of nitrate respiration rather than to the absence of Fnr regulation. As previously mentioned, SOS response in anaerobic conditions only has been reported in *C. glutamicum*[105], which respires nitrates and accumulates nitrite, and *N. gonorrhoeae*[106], which respires nitrite. Dinitrogen trioxide, produced from $NO^{\bullet}$ in the presence of oxygen or by condensation of two molecules of nitrous acid (HNO_2) in anaerobiosis, has been reported to be mutagenic for bacteria[104]. Although at neutral pH the generation of dinitrogen trioxide from nitrous acid is low, due to its low pKa (3.16 at 25 ºC), in TFA, the high concentrations of nitrite generated during anaerobic respiration may lead to the production of enough dinitrogen trioxide to generate DNA damage and the subsequent induction of the SOS response. Additionally, the induction of most of these genes was slow, a kinetic pattern more expected when the accumulation of a toxic and/or mutageninc product or DNA damage is necessary than of a direct regulation by Fnr.

3. Flagellar expression and motility in aerobic and anaerobic conditions in TFA

Sequencing and annotation of TFA genome has also shown that this bacterium has 39 contiguous flagellar genes distributed in 4 RpoN-dependent operons with some internal promoters, apart from the chemotaxis and frimbriae genes. The electron microscopy assays have shown the presence of at least one polar

flagellum in TFA, though the images suggest that this bacterium has a bunch of polar flagella, of which we were unable to determine the number. Flagellar distribution and number has not been yet reported in other *Sphingopyxis* species. Regarding other Sphingomonads, there is high heterogeneity in the flagellar number and distribution. Some of them have been reported to present one polar flagellum, like *Sphingomonas* sp. strain A1[386] and *Sphingosinicella microcystinivorans*[387], others to have lateral flagella, like *Sphingomonas tabacisoli*[388], and others to have a polar bunch of flagella similarly to TFA (lophotrichous flagella), like *Sphingomonas guangdongensis*[389].

Moreover, swimming motility was observed in TFA in aerobic conditions, confirming that these flagellar genes are functional. On the other hand, no swarming motility was observed in TFA in any of the conditions tested, though this was expected as this bacteria didn't show the presence of peritrichous flagella (lateral flagella) in the EM images nor the lateral flagella genes in its sequence[390]. The presence of lateral flagella is not essential for swarming, but most swarming bacteria present them[391].

We analysed the swimming pattern of TFA and we have observed that this bacteria formed bigger swimming halos in minimal medium than in rich medium. Although it has been reported that swimming is a very energy-expensive proccess[392], the fact that TFA is an oligotrophic bacteria could explain that it seems to swim faster in a poorer medium, as it may need lower concentrations of nutrients to be able to sense the gradient and activate its chemotaxis mechanisms. We have also observed the emergence of spontaneous mutants that showed larger swimming circles than the wild type strain. Something similar has been reported in an immotile ΔfleQ mutant of *Pseudomonas fluorescens* that recovered motility after a strong selection by starvation. Genome resequencing of these spontaneous *P. fluorescens* mutants revealed mutations in a distant homolog of *fleQ*, *ntrC*, which allowed it to enhance activation of flagellar genes[393]. The reason of this enhanced motility in TFA spontaneous mutants is still unknown, though transcriptomic analyses might reveal whether the expression of flagellar genes is affected in these mutants, as it happens in those of *P. fluorescens*.

Discussion

We have also characterised the flagellar regulation cascade in TFA. As we have seen, there are different hierarchies of flagellar regulators depending on the bacterium, some of them having four levels of hierarchy while other present only three. TFA seems to have four levels of hierarchy, as we have found 3 possible flagellar-related regulatory proteins in its genome, CtrA, FleQ and FliA, whose mutants were unable to swim. Moreover, the Δ*fleQ* mutant showed no flagella in our EM analyses, as expected. We propose, based on our results, that TFA flagellar hierarchy has CtrA as regulator for class II genes, FleQ as regulator of class III genes, and FliA as regulator of class IV genes. This is a combination of different models of flagellar regulation, as CtrA regulates class II genes in *C. crescentus*[262], FleQ regulates class II genes in *P. aeruginosa*[258] and FliA regulates class III genes in *E. coli* and *S. enterica*[234].

It is interesting the fact that the cell cycle regulator CtrA seems to be involved in flagellar regulation in TFA, which would explain the growth phase-dependence that we have observed in the expression of its flagellins in aerobic conditions. This coordination between the growth phase and the cell cycle has also been described, as previously mentioned, in *C. crescentus*[262], *Magnetospirillum*[263], *R. sphaeroides*[264] and *S. melonis*[265]*.* However, in the case of *C. crescentus*, the reason for this relationship between flagellar regulation and cell cycle is that the division of this bacterium is asymmetrical, giving a swarmer cell and a stalker cell and it is important that every daughter swarmer cell has a flagella[262]. For TFA the reason behind this coordination, however, is still unknown.

Regarding the fimbriae, EM showed that TFA has a bunch of fimbriae in the same pole as the flagella, surrounding them. This bundle formation is typical of type IV pili, though the location of this bundle in the same pole as the flagella has not been yet described in other bacteria. This kind of pili are involved in auto-aggregation, which is consistent with what we have observed in the EM pictures, that TFA cell tended to aggregate with their fimbriae in contact. In pathogen cells these types of pili are also used to adhere to the host cells, but we do not have any evidence that this bacteria could be pathogenic. However, recently, a *S. granuli* has been reported to be a endophytic bacteria that colonises rice[394], so it is possible that this fimbriae could help the bacteria make

contact to the plant host. We have not observed twitching motility in this bacteria, though it should be studied more deeply to be discarded.

In anaerobic conditions we have observed a strong repression of all the flagellar, chemotaxis and fimbriae genes both by dRNA-seq and RT-qPCR assays. We have also observed by swimming assays that the motility of TFA in anaerobic conditions was lost, and by EM analyses that cells barely showed flagella. This is surprising because, as previously mentioned, when bacteria encounter non-favourable conditions they tend to express their flagellar machinery in order to find more favourable environments[188]. However, it is true that in some bacteria a weak repression in anaerobiosis have been observed of some flagellar genes[192,193] and that repression of flagellar genes has also been reported in a stress situation of high nickel concentration[194]. The fact that the *ctrA* gene was repressed in anaerobic conditions could explain the repression of all flagellar genes, being this protein likely the first regulator of the flagellar hierarchy in TFA. However, the over-expression of which we think that could be the second regulator of the hierarchy, *fleQ*, did not restore the capability of TFA to swim in anaerobiosis. This suggests that there may be other genes regulated by CtrA, but not by FleQ, that are essential for the structure or function of TFA flagella, or that regulation of flagellar genes is more complex. At first, we found surprising that growth was not even slightly affected in the Δ*ctrA* mutant, as this protein is involved in cell cycle control. In fact, it has been reported in α-Proteobacteria, that CtrA is essential or presumed-essential for species that evolved from one lineage (*S. meliloti*, *Agrobacterium tumefaciens*, *Brucella abortus*, and *C. crescentus*). However, for bacteria that have evolved from other lineages (*R. capsulatus*, *Silicibacter*, *Magnetospirillum magneticum*, and *Rhodospirillum centenum*), this protein is not essential, probably because of the presence of homologous genes. Moreover, in all of these bacteria, with the exception of the nonmotile *B. abortus*, CtrA plays a role in motility regulation[263]. In the case of fimbriae, we have observed by EM that they are completely lost in anaerobic conditions in almost all the bacteria, with scarce exceptions, as it also happened in Δ*fleQ* mutant, suggesting that they are regulated in the same way as flagella.

Discussion

In the dRNA-seqs comparisons of Δ*narG* and the double Δ*fnr* mutants, the flagellar, pili and chemotaxis genes, which were repressed in anaerobic conditions, lost this repression both in the double Δ*fnr* mutant and in the Δ*narG* mutant, suggesting that the repression of the flagellar genes is not mediated by Fnr. It also does not seem to be a matter of lack of energy, as the expression of these genes is higher in the mutants, where there is no respiration and no energy is being produced. In other conditions where there is carbon source limitation these genes were not repressed either. We still ignore the signal that is leading to the repression of all the motility and chemotaxis machinery in anaerobic conditions in TFA, but repression appears to be exerted through the cell cycle regulator *ctrA*.

CONCLUSIONS

Conclusions

1. *Sphingopyxis granuli* strain TFA is able to grow in anaerobic conditions by respiring nitrate to nitrite, thus becoming the first *Sphingopyxis* strain reported as a facultative anaerobe.

2. TFA is unable to respire nitrite, which is excreted from the cell and accumulated in the culture medium.

3. Part of the nitrite is lost during respiration, probably being transformed in nitric oxide by the nitrate reductase.

4. The mutant in one of the genes coding for a component of the nitrate reductase, *narG*, is unable to grow in anaerobic conditions.

5. Two Fnr type global anaerobic regulators, annotated as FnrN and FixK are involved in anaerobic growth by nitrate respiration.

6. FnrN is more relevant for anaerobic growth than FixK.

7. The recognition sequence of TFA Fnr proteins, Fnr boxes, has been identified and an Fnr regulon of 14 operons has been defined.

8. The central metabolism of TFA is not affected in anaerobic conditions, with scarce exceptions.

Conclusions

9. A great number of genes involved in stress response and SOS DNA repair were upregulated in anaerobic conditions, thus suggesting that anaerobiosis is a hostile and mutagenic environment for TFA.

10. All the flagellar, pili and chemotaxis genes of TFA are repressed in anaerobic conditions and, consequently, this bacterium loses its ability to swim in anaerobiosis.

11. TFA presents lophotricous flagella and a bunch of fimbriae located in the same pole in aerobic conditions. In anaerobiosis TFA loses both bacterial appendages.

12. Spontaneous mutants with increase capacity of swimming can be isolated from strain TFA.

13. Three proteins, CtrA, FleQ and FliA, have been identified as flagellar regulators required for swimming in strain TFA.

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